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# Interactions of Various Pollutants on Causation of Pulmonary Disease

IIT Research Inst, Chicago, IL

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Studies were conducted to determine the effects of single and multiple exposures to individual and pollutant mixtures on resistance to respiratory infections. Results of $NO_2$ studies indicate the greater importance of short-term peak concentrations than prolonged exposures to lower concentrations in increasing the susceptibility to infections. Single 3-hr exposure to mixtures containing various concentrations of $NO_2$ and $O_3$ had an additive effect. Repeated 3-hr exposures for 4 weeks to mixtures consisting of 3760 $\mu g/m^3$ $NO_2$ and 98 $\mu g/m^3$ $O_3$ suggested a synergistic interaction between the two pollutants. Daily 3-hr exposures for 6 months to mixtures of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ resulted in significant excess mortality, and reduced mean survival time in infected animals. Continuation of exposure to the pollutants for 14 days after the infectious challenge resulted in a pronounced increased susceptibility to the respiratory infection, after 1, 2 or 3 month exposure. In non-infected mice this exposure regimen induced changes in the activity of several serum enzymes. Single, multiple or continuous exposures to $SO_2$ in concentration of 13.1 $mg/m^3$ had no effect on susceptibility. A single 3-hour exposure to sulfates indicated that, in decreasing order of effectiveness zinc sulfate, zinc ammonium sulfate and ammonium sulfate reduced resistance to streptococcal pneumonia. The decrease in resistance to the respiratory infection appeared to be related to the zinc ion present in the sulfate complex.

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August 1978

# INTERACTIONS OF VARIOUS POLLUTANTS ON CAUSATION OF PULMONARY DISEASE

by

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Contract No. 68-02-2274

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## FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Studies of health effects of air pollutants have traditionally been concerned with causal association between a single pollutant and a disease state. However, since it is well established that multiple factors are frequently responsible for the occurrence of natural diseases it is important to consider such multiple causality in the evaluation of the biological effects of air pollutants. One of the interactions is depicted by the experimental animal model system which reflects an enhancement of severity of bacterial or viral infections by an exposure to air pollutants. The animal model represents an overall toxic response of the respiratory system, which includes edema, inflammation, cellular necrosis, altered macrophage function and ciliostatis. Thus, this model indicates impairment of the basic defense mechanism in the lung by the combination of exposure to air pollutants and superimposed challenge with airborne infectious micro-organisms. The objective of the project was to determine the effects of single or multiple short-term exposures to various gaseous and particulate pollutants on the resistance to respiratory infections.

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Acting Director,
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## ABSTRACT

Studies were conducted to determine the effects of various pollutants and exposure regimens to the pollutants on the resistance to respiratory infections caused by inhalation of *Streptococcus pyogenes* or influenza virus aerosols. The individual pollutants or their mixtures included nitrogen dioxide ($NO_2$), ozone ($O_3$), sulfur dioxide ($SO_2$), zinc sulfate ($ZnSO_4$), zinc ammonium sulfate ($Zn(NH_4)_2(SO_4)_2$) and ammonium sulfate ($(NH_4)_2SO_4$).

A single 3 hr exposure to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ or to 196 $\mu g/m^3$ (0.1 ppm) $O_3$ increased the susceptibility of mice to streptococcal pneumonia. This was demonstrated by excess deaths and reduced survival time compared to infected mice exposed to filtered air. Within the $NO_2$ concentration range of 2820 (1.5 ppm) to 9400 $\mu g/m^3$ (5.0 ppm) and $O_3$ ranging from 98 (0.05 ppm) to 980 $\mu g/m^3$ (0.5 ppm) the response was linear whereby increase in concentration of the pollutant resulted in increased excess mortalities.

The effect of a single 3 hr exposure to a mixture containing $NO_2$ and $O_3$ was additive. At most concentrations the total excess mortality was equivalent to the sum of excess deaths resulting from exposure to each individual pollutant. At higher concentrations of the pollutant mixture (i.e. 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 980 $\mu g/m^3$ (0.5 ppm) $O_3$ a 1 hr exposure was equally effective as the 3 hr exposure. However, at the lower concentrations the 1 hr exposure was less effective in inducing excess mortality.

The ability to clear inhaled bacteria from lungs was impaired by exposure to the $NO_2$ and $O_3$ mixture. A marked increase in the time required to clear 50% of viable bacteria within 4 to 5 hr after the infectious challenge was seen upon exposure to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture. At the same time a larger percentage of mice exposed to the pollutants had *S. pyogenes* present in their lungs 6 days after the respiratory challenge than mice exposed to filtered air.

A single 3 hr exposure to either 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ or 980 $\mu g/m^3$ (0.5 ppm) $O_3$ but not to a mixture of the two enhanced the susceptibility of mice to influenza infection. Exposure to these pollutants after a respiratory challenge with influenza virus aerosol resulted in excess mortality, reduced survival time and increased lung consolidation.

Daily 3 hr exposures, 5 days/week for 1, 2 or 4 weeks to mixtures consisting of 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ resulted in significant excess mortalities from streptococcal pneumonia. Inasmuch as the exposure to the individual pollutants had no effect on the susceptibility to infection, a syngeristic interaction between the two pollutants appeared to be present. An identical exposure regimen but to 2820 $\mu g/m^3$ (1.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture induced significant excess mortalities only after 4 week exposure. Susceptibility to influenza infection was not affected by the multiple exposure to either of the pollutants or pollutant mixtures.

Daily 3 hr exposures, 5 days/week for 4 weeks to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ did not diminish the effectiveness of influenza vaccine in protecting mice from a respiratory challenge with infectious influenza virus. However, in general more severe lung lesions, increased hemagglutination inhibition (HI) antibody titers and increased rate of HI seroconversion was seen in vaccinated mice exposed to the pollutants.

To more closely simulate ambient environmental exposure of human populations, studies were conducted using five different exposure regimens, three of which were of major importance.

- daily 3 hr exposure, 5 days/week to 940 $\mu g/m^3$ (0.5 ppm) $NO_2$
- daily 3 hr exposure, 5 days/week to a mixture of 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$
- continuous exposure 24 hr/day, 7 days/week to 188 $\mu g/m^3$ (0.1 ppm) $NO_2$ with superimposed daily 3 hr peaks, 5 days/week of 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$

The 6 month exposure to any of the pollutant combinations resulted in a marked increase in the susceptibility to streptococcal pneumonia. This was manifested by excess mortality and reduced survival time. The increase in susceptibility was much more pronounced after 1, 2 or 3 months when the animals continued to be exposed for 14 days after the infectious challenge to the pollutants instead to filtered air.

After a 3 month exposure to the $NO_2$ and $O_3$ mixtures there was a marked decrease in the ability of the animals to clear inhaled *S. pyogenes* from their lungs. This impairment was not present in mice exposed daily for 3 hr to 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ only. Pulmonary cellular defenses were somewhat affected by the 3 month exposure to the pollutant mixture but not by the daily exposure to $NO_2$ only. The total cell count in the fluid lavaged from the lungs was markedly reduced as was the viability and phagocytic activity of the alveolar macrophages.

In non-infected mice the 6 month continuous exposure to 188 $\mu g/m^3$ (0.1 ppm) $NO_2$ with the superimposed daily 3 hr $NO_2$ and $O_3$ mixture peaks resulted in significant decrease in serum cholinesterase, increase in serum glutamic pyruvic transaminase and serum glutamic oxalic transaminease and shift to

lactate dehydrogenase (LDH-1) isoenzyme. In the same treatment group a decrease in hematocrit and an increase in platelet counts was seen. A similar trend in changes was seen in mice daily exposed for 3 hr to the $NO_2$ and $O_3$ mixture only. The changes in blood serum enzyme activity were in close agreement with those reported for guinea pigs exposed for 4 months to 940 $\mu g/m^3$ (0.5 ppm) $NO_2$.

Single or up to 15 multiple daily 3 hr exposures to 13.1 $mg/m^3$ (5.0 ppm) $SO_2$ alone or in mixture with either, carbon particles, zinc sulfate particles or $NO_2$ and $O_3$ had no effect on the susceptibility of mice to streptococcal pneumonia. Continuous exposure 24 hr/day for up to 3 month to the same $SO_2$ concentration also had little effect on the susceptibility to respiratory infections as well as on other health effect parameters.

Inhalation of zinc sulfate (>1.2 $mg/m^3$) or zinc ammonium sulfate (>2.1 $mg/m^3$) followed by a respiratory challenge with *S. pyogenes* aerosol resulted in significant excess mortality and reduced survival time in mice. For the 3 hr inhalation exposure the estimated concentration of zinc sulfate which induced 20% excess mortality ($ED_{20}$) was 1.45 $mg/m^3$ whereas that of zinc ammonium sulfate was 2.40 $mg/m^3$. Exposure to ammonium sulfate aerosol in concentrations ranging from 1.1 to 5.3 $mg/m^3$ had no effect on susceptibility to streptococcal pneumonia.

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## ABBREVIATIONS

| | |
|---|---|
| AlP | -- alkaline phosphatase |
| AM | -- alveolar macrophages |
| AP | -- acid phosphatase |
| CHE | -- cholinesterase |
| D/T | -- dead/total |
| ED | -- effective dose |
| HBDH | -- hydroxybutyrate dehydrogenase |
| HI | -- hemagglutination-inhibition antibody |
| hr | -- hour |
| ICDH | -- isocitric dehydrogenase |
| LD | -- lethal dose |
| LDH | -- lactate dehydrogenase |
| MMD | -- mass median diameter |
| MST | -- mean survival time, days |
| $m^3$ | -- cubic meter |
| µg | -- microgram, 1 µg = $10^{-6}$g |
| $NO_2$ | -- nitrogen dioxide (ppm x 1880 = µg/$m^3$) |
| $(NH_4)_2SO_4$ | -- ammonium sulfate |
| $O_3$ | -- ozone (ppm x 1960 = µg/$m^3$) |
| ppm | -- parts per million |
| RH | -- relative humidity |
| SGOT | -- serum glutamic oxaloacetic transaminase |
| SGPT | -- serum glutamic pyruvic transaminase |
| $SO_2$ | -- sulfur dioxide (ppm x 2620 = µg/$m^3$) |
| $t_{50}$ | -- time (hr) to clear 50% of inhaled bacteria from lungs |
| $Zn(NH_4)_2(SO_4)_2$ | -- zinc ammonium sulfate |
| $ZnSO_4$ | -- zinc sulfate |

# SECTION 1

# INTRODUCTION

Studies of health effects of air pollutants have traditionally been concerned with causal association between a single pollutant and a disease state. However, since it is well established that multiple factors are frequently responsible for the occurrence of natural diseases it is important to consider such multiple causality in the evaluation of the biological effects of air pollutants. One of the interactions is depicted by the experimental animal model system which reflects an enhancement of severity of bacterial or viral infections by an exposure to air pollutants. The animal model represents an overall toxic response of the respiratory system, which includes edema, inflammation, cellular necrosis, altered macrophage function and ciliostatis. Thus, this model indicates impairment of the basic defense mechanism in the lung by the combination of exposure to air pollutants and superimposed challenge with airborne infectious microorganisms.

Results of experimental studies which utilized this animal model clearly demonstrated that inhalation of either ozone or nitrogen dioxide significantly enhances the susceptibility to bacterial pneumonias. On the other hand, only sparse data are available on the effect of exposure to mixture of these two pollutants or to other pollutants on the resistance to respiratory infection. It is well recognized that effects of air pollutants may be additive or synergistic with each other. Such combined effects can be ascribed to a variety of factors. For example, one pollutant can affect the site of the deposition of another, a pollutant can affect the lung clearance mechanism so that upon inhalation the second pollutant cannot be removed, or a pollutant can produce an effect in the lung which makes it more vulnerable to the effects of the second pollutant. Occasionally the type of interaction can be predicted on the basis of chemical composition of each of the components of the pollutant mixture. More often, however, it is not possible to forecast effects of the mixture and therefore empirical studies must be carried out.

The objective of the project was to determine the effects of single or multiple short-term exposures to various pollutants on the resistance to respiratory infections. The studies were conducted using mice as the experimental animal host, exposed for 3 hours to either ozone, nitrogen dioxide, sulfur dioxide, various sulfates as well as mixtures containing various concentrations of these pollutants, and then challenged with *Streptococcus pyogenes* aerosol. Extensive studies were also carried out to define the effects of daily 3-hr peak exposure to nitrogen dioxide and ozone mixtures on the resistance to infection and other health parameters.

Results of these studies were sumamrized in a series of quarterly progress reports (IITRI Report No. L6089-1 through L6089-7). Phases of the studies were reported at the International Conference on Photochemical Oxidant Pollution and Its Control, September 12-17, 1976, Raleigh, NC and several Environmental Protection Agency workshops. Publications resulting from the studies are:

Effects of Short-Term Inhalation of Nitrogen Dioxide and Ozone Mixture
R. Ehrlich, J. C. Findlay, J. D. Fenters and D. E. Gardner.
Environmental Research 14, 223-231, 1977.

Susceptibility to Bacterial Pneumonia of Animals Exposed to Sulfates
R. Ehrlich, J. C. Findlay and D. E. Gardner.
Toxicology Letters (accepted for publication).

## SECTION 2

## CONCLUSIONS

Studies were conducted to determine the effects of single and multiple 3 hr exposures to individual air pollutants and pollutant mixtures on the resistance to respiratory infections. The results reconfirm the utility and applicability of this experimental animal model system to studies of the effects of air pollutants in concentrations approximating those found in ambient environment.

Results of nitrogen dioxide ($NO_2$) studies point out to the greater importance of short-term exposures to peak concentrations than prolonged exposures to low concentrations of $NO_2$ in increasing the susceptibility to streptococcal pneumonia. A single 3 hr exposure to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ or a daily 3 hr exposure, 5 days/week for 6 months to 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ resulted in significant excess mortality and reduced survival time in mice infected with *Streptococcus pyogenes* aerosol. The data reemphasize the necessity for the establishment of primary air quality standard for short-term exposure to $NO_2$. The current air quality standard of 100 $\mu g/m^3$ (0.05 ppm) $NO_2$ represents an annual arithmetic average which does not take into consideration the health effects of short-term exposures to elevated concentrations of this pollutant.

Single 3-hr exposure to mixtures containing various concentrations of $NO_2$ and ozone ($O_3$) had an additive effect. The excess mortalities due to streptococcal pneumonia in mice exposed to such mixtures were equivalent to the sum of excess death resulting from exposure to each individual pollutant. Repeated daily 3-hr exposures for up to 4 weeks to mixtures consisting of 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ suggested the presence of a synergistic interaction between the two pollutants in increasing the susceptibility to respiratory infection.

Daily 3 hr exposures, 5 days/week for 6 months to mixtures of 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ resulted in significant excess mortality, and reduced mean survival time in infected animals. Continuation of exposure to the pollutants for 14 days after the infectious challenge resulted in a pronounced increased susceptibility to the respiratory infection, after 1, 2 or 3 month exposure. In non-infected mice this exposure regimen induced changes in the activity of several blood serum enzymes.

Single or multiple 3 hr exposures as well as continuous exposures to sulfur dioxide ($SO_2$) in concentration of 13.1 $mg/m^3$ (5.0 ppm) had no marked effect on the susceptibility to bacterial pneumonia.

A single 3 hr exposure to sulfates indicated that, in decreasing order of effectiveness zinc sulfate, zinc ammonium sulfate and ammonium sulfate reduced resistance to streptococcal pneumonia. The decrease in resistance to the respiratory infection appeared to be related to the zinc ion present in the sulfate complex. The lesser effect of zinc ammonium sulfate than zinc sulfate in enhancing the severity of the infection and the absences of any effect due to inhalation of ammonium sulfate might be due to the presence of the ammonium ion which could possibly neutralize the effectiveness of the metallic cation. These studies indicate that resistance to respiratory infection can be used as a sensitive parameter for evaluation and ranking of health effects of sulfates. Moreover, the differences in response to inhalation of the three sulfates suggest the advisability of identification of the molecular composition of sulfate particles during air monitoring. This is of special importance when the air monitoring is conducted as part of epidemiological studies of health effects of environmental pollution.

## SECTION 3

## RECOMMENDATIONS

Studies of the effects of exposure to air pollutants on the resistance to respiratory infections represent a sensitive and realistic approach to defining the health effects of pollutants. Thus, studies should continue to more precisely define the causal relationship between inhalation of air pollutants and acute respiratory infections in laboratory animals. This animal model should be used to establish a toxic ranking of various pollutants especially those within a given group of related pollutants.

To simulate environmental pollution conditions studies should include the use of mixture of gaseous as well as particulate pollutants. Exposures to additional stresses, such as exercise or extremes of temperature and humidity should also be included in such investigations.

Since man is usually exposed to low concentrations of pollutants with superimposed cyclical higher concentrations, similar exposure regimens should be used during long-term exposure studies. This is especially important in studies of pollutants such as nitrogen dioxide, where within a given exposure regimen the concentration is of greater importance than the duration of exposure.

Additional studies should also be carried out to elucidate the mechanisms by which the defense systems against respiratory infections are affected by exposure to the pollutants. Thus, the cellular and humoral immunity as well as the activity of alveolar macrophages in several species of laboratory animals must be investigated.

## SECTION 4

## MATERIALS AND METHODS

### ANIMALS

The strains of mice used in the various experiments included female CF-1 (ARS, Madison, WI) and CD2F1 (Murphy Laboratory, Plainfield, IN), and male and female BDF1 (Laboratory Supply, Indianapolis, IN; Murphy Laboratory). The 5-to-8 week old mice were quarantined for 7-14 days before being used in the studies. During the quarantine period and throughout the experiments the mice were housed in groups of 8 in stainless steel shoe box cages. Food and water were provided, *ad libitum*.

### EXPOSURE CHAMBERS

The 432-liter capacity (120 x 60 x 60 cm) chambers used for short-term exposure to air pollutants were constructed from plexiglass. During the short-term exposures the mice were housed individually in separate compartments of specially designed stainless steel wire cages. The pollutants entered the chambers from the top of one side at the approximate rate of 60 ± 5 liters/min and were exhausted at the top of the opposite side. To assure a homogeneous distribution of the pollutants a small fan was operated continuously in each chamber. To prevent a build-up of ammonia during the exposure of animals to the pollutants, deotized cage board (Upjohn Co., Kalamazoo, MI) was placed on the floor of each chamber.

Temperature in the exposure chambers was maintained at 24 ± 2°C at ambient humidity (~40% RH). During experiments designed to study the effects of elevated humidity, pressurized steam was introduced into the chambers after it passed through condensation traps. Continuous reading of the relative humidity was provided by Airguide Humidity Indicator (Model 605) located in each chamber. An electro-hygometer (Lab-line Instruments, Inc., Melrose Park, IL) was used to check and calibrate the humidity indicators.

The compressed air supplied to the exposure chambers was passed first through an Alemite filter (Model 7620, Steward Warner, Chicago, IL) to dry the air and to remove traces of oil. Before entering the individual exposure chambers the air was further purified by passage through disposable air purifier and flow equalizer (Koby Inc., Marlboro, MA).

For long-term exposures to the pollutants, five identical 4420-liter aluminum-lined chambers 1.2 x 1.8 x 2.0 m were used. Randomly selected mice were housed in suspended wire cages, which were rotated 2 to 3 times/week to various positions on the cage racks to assure unbiased exposure to the experimental environment. The pollutants diluted in charcoal-filtered air entered the chambers at a rate of approximately 20 changes per hr. The same air flow pattern was maintained in the control chamber, where ambient air

passed through charcoal filters, was used. The mean temperature in the chambers was 24° ± 2°C at ambient humidity (~30% RH). To verify the concentration and homogeneity of the pollutants, air samples were taken from different sections of the chambers. All exposure chambers were connected to a manifold system capable of manual or automatic sampling. When the automatic arrangement was used, the sampling time for each chamber was 15 min.

POLLUTANTS

Nitrogen Dioxide ($NO_2$)

A 1 or 5% $NO_2$ gas mixture in balanced air, >99.5% pure, (Matteson, Joliet, IL) was diluted with the filtered air in a mixing chamber and then passed into the animal exposure chambers. The $NO_2$ concentration was monitored continuously by a NO-$NO_2$-$NO_x$ chemiluminescent analyzer (Model 8101 B. Bendix Corp., Ronceverte, WV) and was expressed in ppm and $\mu g/m^3$ (ppm x 1880 = $\mu g/m^3$).

Ozone ($O_3$)

A high-voltage generator (IITRI) was used to convert filtered air or oxygen to $O_3$. To provide the desired concentration, $O_3$ was diluted with filtered air in a glass mixing chamber and then passed into the animal exposure chambers. The $O_3$ concentration was monitored continuously with an $O_3$ chemiluminescent analyzer (Model OA 310, Meloy Laboratories, Springfield, VA) and was expressed in ppm and $\mu g/m^3$ (ppm x 1960 = $\mu g/m^3$).

$NO_2$-$O_3$ Mixture

Each gas was introduced into separate glass vessel and then combined in a glass mixing chamber. The $NO_2$ and $O_3$ mixture was then passed into the animal exposure chambers. The concentration of each gas in the exposure chamber was monitored continuously with the $NO_x$ and $O_3$ chemiluminescent analyzers.

Sulfur Dioxide ($SO_2$)

A 1 or 5% $CO_2$ gas mixture in balanced air, >99.98% pure, (Matteson, Joliet, IL) was diluted with filtered air in a glass mixing chamber and then passed into the animal exposure chambers. The $SO_2$ concentration was monitored continuously with a total sulfur analyzer (Model 8300, Bendix Corp., Ronceverte, WV). The analyzer was connected to a dilution panel (Bendix Corp.) equipped with a stainless steel capillary, metering regulator, pull-to-test diverter valve and diluting manifold. The gas sample was diluted 10-fold with filtered air before being analyzed. Periodically the $SO_2$ concentration in the chamber was monitored by the pararosaline method (1). Air samples for the assays were collected in a all-glass impinger (AGI) operated for 30 min at a rate of 1 liter/min. The concentration of $SO_2$ was expressed in ppm and $\mu g/m^3$ (ppm x 2620 = $\mu g/m^3$).

## Carbon Particles

The carbon (Carbon black, 99.5% pure, Sterling MT CT-6729, Cabot Corp., Boston, MA) aerosol was generated by a Wright Dust Feeder (L. Adams Ltd., London, England). A slowly rotating scraper blade continuously released a small quantity of carbon powder into an air stream passed through a nozzle with an impaction plate to break the agglomerates. Particles larger than 5μ were removed by sedimentation during passage of the aerosol through a 30 cm long settling chamber before entering the mixing chamber.

The average mass concentration of carbon in the aerosol was estimated by gravimetric analysis. The particles were collected on a HA 0.45μ Millipore filter backed by a 0.22μ Millipore filter, both 47 mm in diameter. The total air collected for assay during the 3 hr animal exposures ranged from 210 to 350 liters. The concentration of carbon particles per cubic meter of air was estimated by dividing the weight of the particles collected on the membrane filters by the total volume of collected air.

## Sulfates

Aqueous dilutions of zinc sulfate ($ZnSO_4$, 99.4% pure, Baker Chem., Phillisburg, NJ), ammonium sulfate ($(NH_4)_2SO_4$, 99.7% pure, Fisher, Chicago, IL), and zinc ammonium sulfate ($Zn(NH_4)_2(SO_4)$, 95.0% pure, ICN Pharmaceuticals, Plainview, NY) were used to generate the aerosol by using a DeVilbiss Model 40 nebulizer. Depending on the desired concentration of the sulfate, the nebulizer was operated at pressures ranging from 2 to 6 psi. The sulfate aerosol was passed through a heated glass tube into a glass mixing vessel where the particles were diluted with dried filtered air and then introduced into the animal exposure chamber.

The mass median size of droplets produced by the DeVilbiss 40 was 3 μm (2), The estimated mass median diameter of the evaporated sulfate aerosol particles was 0.63 μm and the count median diameter approximately 0.31 μm. For particle size analysis aerosol samples were collected through a 7 μm single-stage preimpactor on a methanol-cleaned 25 mm glass discs coated with a 50% glycerol-water solution. Microscopic examination indicated that approximately 90% of the airborne particles were <3 μm in diameter.

For quantitative assay, the airborne sulfate particles in the chamber were collected on membrane filter (0.22 μM) at a flow rate of 1.5 to 3.0 liters/min. After collection the filteres were refluxed with 20 ml of distilled water for 90 min. The extract was treated with barium chloride to form barium sulfate and the turbidity was measured against a standard curve. The concentration of sulfate was expressed as $mg/m^3$ of $SO_4$ (3).

## INFECTIOUS AGENTS

### Bacteria

*Streptococcus pyogenes* (Lancefield Group C) was used for the respiratory infectious challenge. To maintain the stock culture, mice were injected intraperitoneally with a suspension of *S. pyogenes* and killed 24 hr later. Heart blood was incubated on blood agar for 48 hr at 37°C and *S. pyogenes* colonies isolated from the agar were inoculated in Todd Hewitt broth (BBL). After 18 hr incubation at 37°C the bacteria were harvested in approximately 1 ml aliquots and frozen at -70°C. For aerosol dissemination, 0.8 ml of the thawed bacteria was grown in Todd Hewitt broth for 18 hr at 37°C. The optical density of the culture was adjusted in 0.1% peptone-water to the appropriate density as measured at 440 Mμ in Spectronic 20 densitometer.

### Virus

Mouse-adapted influenza virus was passaged several times in mice and a 20% lung suspension of the virus was prepared and frozen at -70°C. The virus was identified by use of specific antiserum obtained from the National Institutes of Health. For aerosol dissemination, the virus was appropriately diluted in 0.2% bovine albumin containing 100 units/ml of Polymyxin and 100 units/ml of Neomycin.

## INFECTIOUS CHALLENGE

Infectious respiratory challenge was performed in a 400-liter plexiglass aerosol chamber (71 x 61 x 92 cm) contained within a microbiological safety cabinet. Temperature and humidity in the aerosol chamber were maintained at 24 ± 2°C and 65 ± 5% RH. A continuous flow DeVilbiss nebulizer (Model 841) was used to produce the bacterial aerosol. Filtered air was supplied to the inlet of the nebulizer at a flow rate of approximately 8 liters/min. For the infectious challenge, mice were housed in individual compartments of a stainless steel wire cage and exposed to the aerosol for 10 to 20 min. After the challenge, the mice were removed from the chamber and housed in conventional cages in a clean-air, isolated animal room.

The inhaled dose of bacteria was determined by killing three mice immediately after the infectious challenge. The lungs were removed, weighed, and homogenized in 1.8 ml of sterile 0.1% peptone water. The suspension was diluted, plated on blood agar and the colonies counted after 48 hr incubation at 37°C. The inhaled dose generally ranged from 5-25 x $10^3$ viable bacteria per gram of lung tissue.

## HEALTH EFFECTS PARAMETERS

### Mortality and Survival Time

Deaths were recorded daily during the 14-day observation period following the infectious challenge. The mortality rates were reported as either number of dead out of the total mice used (D/T), percent mortality (%) or percent excess mortality (% deaths pollutants - % deaths filtered-air controls).

The mean survival time was calculated according to the following equation

$$MST = \frac{(AB)+(dL)}{n}$$

where A is the last day on which any individual mouse was alive; B is the number of mice surviving A days; d is the last day of observation; L is the number of mice alive on day d; and n is the total number of mice used in the experiment.

Lung Clearance of Inhaled Bacteria

Groups of five mice were killed immediately after the respiratory challenge with *S. pyogenes* aerosol. The lungs were removed aseptically, weighed, homogenized and cultured quantitatively by using the procedure described for the determination of the inhaled dose. These initial counts (0 hr), calculated as the number of viable bacteria per gram of lung, were considered as unity (100%). Thereafter groups of five mice were killed at 1,2,3,4 and 5 hr and, as appropriate, at 1,2,3 and 6 days after the respiratory challenge and their lungs assayed in an identical manner. The counts were calculated as percent recovery of these present at the 0 hr.

The rate of clearance was expressed as $t_{50}$ i.e., time in hours required for 50% of the inhaled viable bacteria to be cleared from the lungs. The $t_{50}$ was determined on a semilogarithmic regression after converting the percent recovery to logarithmic values.

Phagocytic Activity

Mice were killed by intraperitoneal injection of sodium pentobarbital and the lung-heart-trachea complex was removed from the chest cavity *in toto*. A blunted hypodermic needle inserted into the trachea and fastened with surgical suture was used to lavage the lungs by repeated infusions of warm (37°C) saline. Alveolar macrophages were isolated from the lavage fluids by centrifugation at 365 xg and washing in Hanks medium.

Total cell counts were made in a hemocytometer. Differential counts were made on air-dried smears of cells fixed in methanol and stained with Wright's stain to determine the cellular distribution (i.e., percent of alveolar macrophages, polymorphonuclear leucocytes and lymphocytes). Viability of the macrophages was determined by dye exclusion using 1% trypan blue.

Lung Edema

To detect the extent of edema, groups of 8 to 10 mice were killed and individual lungs weighed immediately after removal. The lungs were then dried in a vacuum dessicator and reweighed at 24 hr intervals until no additional weight loss was apparent. The extent of lung edema was expressed as the wet-to-dry weight ratio.

## Lung Consolidation

The extent of pulmonary lesions after challenge with influenza virus was expressed as the percentage of the total lung consolidated (4). A score of 1 represented 25% lung consolidation, 2 = 50%, 3 = 75%, 4 = 100%, and a score of 5 was assigned to dead animals that succumbed to the infection.

## Serological Assays

The hemagglutination inhibition (HI) antibody levels were determined in duplicate by the microtiter method in disposable "V" plates. In all HI tests, 1% chicken red blood cells and four hemagglutinating units of egg-adapted influenza $A_2$/Taiwan virus were used. Type $A_2$ influenza (Taiwan/1/64, NIH) antiserum served as the positive control. To remove nonspecific inhibitors of hemagglutination the sera were heat inactivated at 56°C for 30 min and treated with trypsin-periodate. The primary HI antibody titer in all mice was <8.

## Scanning Electron Microscopy (SEM) and Histopathology

Mice were anesthetized by an intraperitoneal injection of pentobarbital and the abdomen was opened on the midline, permitting access to the ventral aorta. The aorta was severed, the animal exsanguinated, the chest was opened, and the lungs and trachea removed *in toto*. The trachea was cannulated to the level of the first cartilaginous ring and the lungs were expanded with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative at 20 cm water pressure. After a 2 hr perfusion with the lungs completely immersed in the fixative, the trachea was ligated and the lungs floated in fixative. The trachea and main stem bronchi were isolated from the lungs. The lungs were sectioned with a razor blade so as to reveal the bronchus and alveoli of each lobe.

After cannulation of the trachea, the head was removed, the skin was retracted and the lower jaw removed by sectioning through the ramus. The cranium was cut off immediately posterior to the orbits, leaving only the nasal cavity. The nasal bones were reflected utilizing small forceps and the cavity sectioned in half by inserting a razor blade on one side of the median septum and severing the hard palate.

All tissues were washed in distilled water, and dehydrated with increasing concentration of alcohol. Pentyl acetate was then substituted for the alcohol, and the tissues were critical point dried in carbon dioxide. The dried trachea was sectioned longitudinally, and all tissues were cemented to stubs, gold coated in a Denton vacuum evaporator and examined in a Kent-Cambridge Mark II Stereoscan scanning electron microscope at 20 kV.

Lung and trachea tissues were also processed for conventional histopathological examination. Sections were cut at 5μ, stained with hematoxylin and eosin and examined microscopically.

## Hematology Tests

Erythrocytes and leukocytes were counted in duplicate in a Coulter Electronic Particle Counter with 100 aperature. Reference blood samples were counted for standardization (5). Hematocrit was determined in capillary tubes using a microcapillary head centrifuge. Hemoglobin was measured as cyanomethemoglobin, with a reference solution as standard (6). Wright's stain was used for differential leukocyte counts. Reticulocytes were counted by the new methylene blue N method (7). Platelets were counted in a hemocytometer with a phase microscope.

## Clinical Chemistry Tests

The clinical chemistry tests were run on the day of blood collection, except in a few instances where storage of serum at -20°C was necessary. The tests on the sera were performed on a centrifugal analyzer (Union Carbide, Tarrytown, NY) and included alkaline phosphatase (AlP) (8), lactic dehydrogenase-L (LDH) (9), hydroxybutyrate dehydrogenase (HBDH) (10), isocitric dehydrogenase (ICDH) (11), glutamic oxalic transaminase (SGOT) (12), glutamic pyruvic transaminase (SGPT) (12), cholinesterase (CHE) (13) and acid phosphatase (AP) (14). The lactic dehydrogenase isoenzymes were determined in a Beckman microzone electrophoresis system.

## STATISTICAL ANALYSES

The significance of the differences in mortality rates was determined by the Chi-square ($X^2$) test with a 2 x 2 contingency table. The Student t test, analysis of variance, and least square linear regression were applied to the data as appropriate.

## SECTION 5

## RESULTS AND DISCUSSION

### SINGLE 3-HR EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES

#### Mortality and Survival Time

Results of previous studies conducted in our laboratories (15) indicated that a single 2 hr exposure of mice to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ significantly enhanced the mortality resulting from a superimposed bacterial pneumonia initiated by inhalation of airborne *Klebsiella pneumoniae*. Coffin and Gardner (16) reported a similar enhancement of mortality after a 3 hr exposure to 157 $\mu g/m^3$ (0.08 ppm) $O_3$ and challenge with *Streptococcus* sp. aerosol. To provide unified data in terms of duration of exposure and the infectious agent, mice were exposed for 3 hr to various concentrations of $NO_2$ and $O_3$ and then challenged with *S. pyogenes* aerosol. The death rates obtained during numerous replicate exposures to the pollutants are summarized in Figure 1. A significant increase in mortality rates over those in control mice was seen upon the 3 hr exposure to 196 $\mu g/m^3$ (0.1 ppm) $O_3$ or 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$. Moreover, a linear relationship was present between the concentration of the pollutants and mortality rates with a correlation coefficient (r) of 0.969 for $NO_2$ and 0.996 for $O_3$.

To determine the effects of inhalation of air pollutant mixtures, groups of mice were exposed to various concentrations of $NO_2$, $O_3$, $NO_2$ and $O_3$ mixture or to filtered air. The four treatment groups, usually consisting of 24 mice each, were then simultaneously challenged with *S. pyogenes* aerosol. The mortality rates were initially compared on the basis of individual exposure experiments and results of replicate experiments were pooled for further statistical analysis. The excess mortalities based on a minimum of four replicate experiments at each concentration of the pollutants are summarized in Table 1. In most instances the excess mortality was equivalent to the sum of those induced by inhalation of each individual pollutant. Thus, the effect of the 3-hr inhalation of $NO_2$ and $O_3$ mixtures was additive.

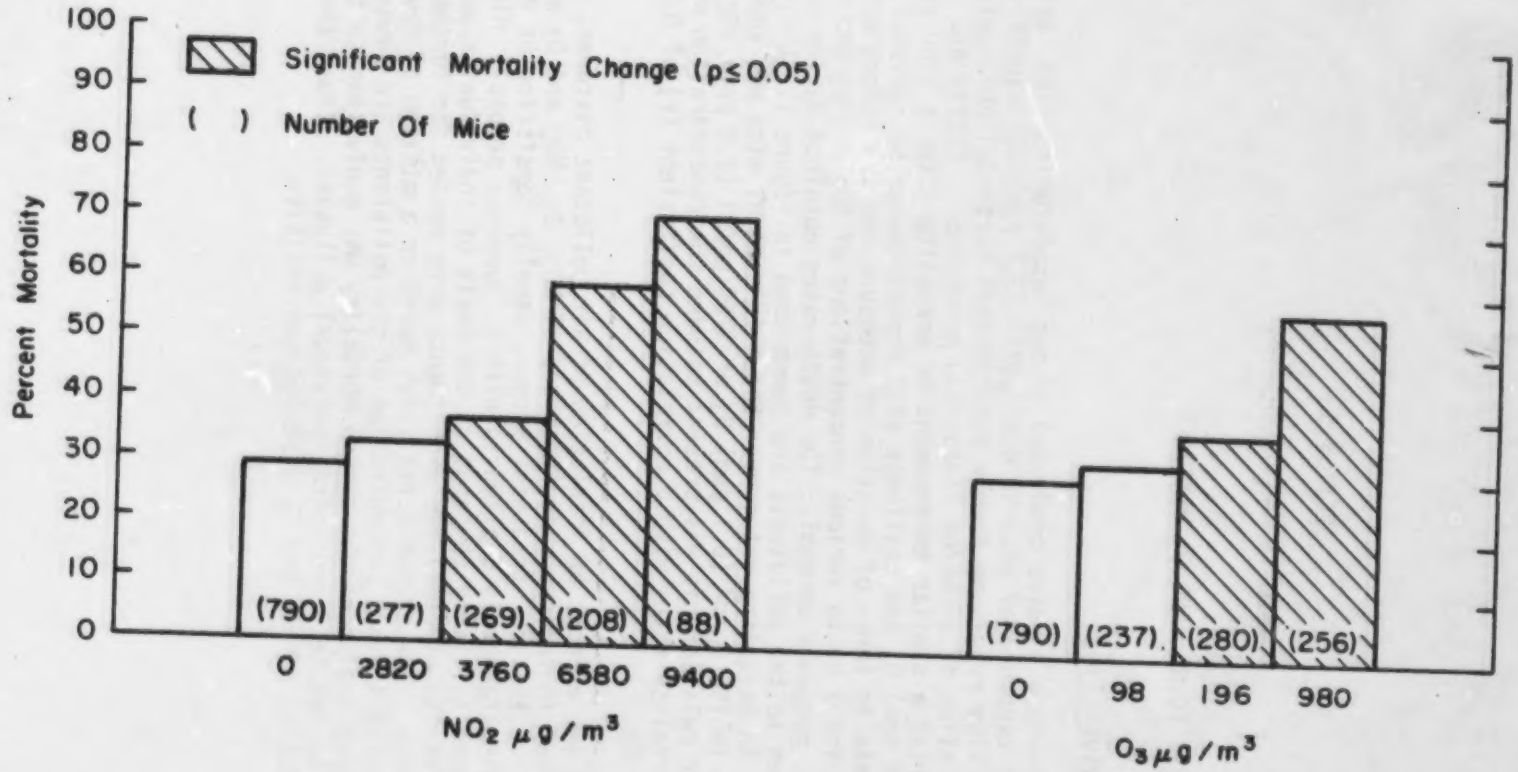


Figure 1. Percent mortality in mice exposed for 3 hr to various concentrations of $NO_2$ or $O_3$ and challenged with Streptococcus aerosol.

TABLE 1. EXCESS MORTALITY IN MICE CHALLENGED WITH *STREPTOCOCCUS* AEROSOL AFTER SINGLE 3-HR EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES

| Concn. ($\mu g/m^3$) $NO_2$ \ $O_3$ | Excess Mortality (%) (Pollutant - Control) 0 | 98 | 196 | 980 |
|---|---|---|---|---|
| 0 | 0 | 5.4 | 7.2 | 28.6* |
| 2820 | -1.7 | 4.6 | 4.2 | 23.9* |
| 3760 | 14.3* | 22.0* | 9.1* | 56.2* |
| 6580 | 28.2* | - | 38.5* | 68.7* |
| 9400 | 35.7* | - | - | 65.3* |

* Significant change from corresponding infected mice exposed to filtered air ($p \leq 0.05$).

The mean survival time was also affected by the exposures to the individual pollutants as well as the pollutant mixtures. Noninfected mice exposed to only the pollutants survived the 14 day observation period, whereas the mean survival time of infected mice, not exposed to the pollutants, was 12 days. Significant shortening of survival time was observed upon exposure to 6580 (3.5 ppm) or 9400 $\mu g/m^3$ (5 ppm) $NO_2$ or 980 $\mu g/m^3$ (0.5 ppm) $O_3$. Although the single 3 hr exposure to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ or 196 $\mu g/m^3$ (0.1 ppm) $O_3$ significantly enhanced the mortality rates no apparent effect on the course of the infection was noted. Shortened survival time was also seen upon exposures to all mixtures containing 980 $\mu g/m^3$ (0.5 ppm) $O_3$ irrespective of concentration of $NO_2$, and to the mixture containing 6580 $mg/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$.

An exploratory experiment was conducted to determine whether a 1-hr exposure to the pollutants induces a change in resistance to streptococcal pneumonia similar to that observed upon a 3-hr exposure. As seen in Table 2 the 3-hr exposure to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ resulted in a significant increase in mortality and decrease in the survival time. However, a 1 hr exposure to the same $NO_2$ concentration failed to produce any marked changes. Inhalation of 980 $\mu g/m^3$ (0.5 ppm) $O_3$ or a mixture consisting of 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 980 $\mu g/m^3$ (0.5 ppm) $O_3$ resulted in significant excess mortality and decrease in survival time, irrespective of the duration of the exposure. The 3-hr exposure to the $NO_2$ and $O_3$ mixtures at the two lower concentrations also resulted in a marked enhancement of mortalities. However, this was not seen upon 1 hr exposure to the mixture of the pollutants.

TABLE 2. MORTALITY AND SURVIVAL RATES OF MICE EXPOSED FOR 3 OR 1 HR TO $NO_2$ AND $O_3$ MIXTURES AND CHALLENGED WITH *STREPTOCOCCUS* AEROSOL

| Concn., $\mu g/m^3$ | | Duration of Exposure, hr | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 3 | | | | 1 | | | |
| | | Mortality | | | | Mortality | | | |
| $NO_2$ | $O_3$ | D/T | % | Change % | MST, day | D/T | % | Change % | MST, day |
| 0 | 0 | 3/36 | 8.3 | - | 13.5 | 10/72 | 13.9 | - | 12.9 |
| 6580 | 0 | 8/36 | 22.2 | +13.9** | 12.2* | 8/72 | 11.1 | - 2.8 | 13.1 |
| 0 | 980 | 19/36 | 52.8 | +44.5* | 10.1* | 18/72 | 25.0 | +11.1** | 12.0** |
| 6580 | 980 | 26/36 | 72.2 | +63.9* | 8.2* | 27/72 | 37.5 | +23.6* | 11.1* |
| 0 | 0 | 1/44 | 2.3 | - | 13.8 | 4/68 | 5.9 | - | 13.7 |
| 3760 | 0 | 1/44 | 2.3 | 0 | 13.9 | 5/96 | 5.2 | - 0.7 | 13.6 |
| 0 | 196 | 1/44 | 2.3 | 0 | 13.8 | 6/96 | 6.3 | + 0.4 | 13.5 |
| 3760 | 196 | 6/44 | 13.6 | +10.7* | 12.8 | 10/96 | 10.4 | + 4.5 | 13.1 |
| 0 | 0 | 4/24 | 16.7 | - | 12.5 | 7/48 | 14.6 | - | 12.8 |
| 2820 | 0 | 3/24 | 12.5 | - 4.2 | 13.1 | 7/48 | 14.6 | 0 | 12.9 |
| 0 | 98 | 3/24 | 12.5 | - 4.2 | 13.0 | 4/48 | 8.3 | - 6.3 | 13.4 |
| 2820 | 98 | 8/24 | 33.3 | +16.6 | 12.0 | 3/48 | 6.3 | - 6.3 | 13.5 |

Significant change from corresponding infected mice exposed to filtered air.

* ($p \leq 0.05$)

** ($p \leq 0.1$)

The inability to establish the statistical significance of the increased mortality upon exposure to 2820 $\mu g/m^3$ (1.5 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ mixture appears to be due to the small number of animals used in this experiment. The data suggest that a single 1-hr exposure to the individual pollutants as well as a mixture of the pollutants is less effective in altering the resistance to streptococcal pneumonia than a 3 hr exposure.

A single experiment was conducted to determine the susceptibility of mice challenged with $A_2$/Taiwan influenza virus followed 24 hr later by a single 3 hr exposure to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$, 980 $\mu g/m^3$ (0.5 ppm) $O_3$ or a mixture containing the same $NO_2$ and $O_3$ concentrations. Only exposure to $NO_2$ resulted in significant excess mortality, increase in lung consolidation and reduced survival time (Table 3). Although mortality was almost twice as high among mice exposed to $O_3$ than infected mice exposed to filtered air, the difference was not significant.

TABLE 3. CHANGES IN RESISTANCE IN MICE CHALLENGED WITH $A_2$/TAIWAN INFLUENZA VIRUS FOLLOWED 24 HR LATER WITH A 3 HR $NO_2$-$O_3$ EXPOSURE

| Concn., $\mu g/m^3$ | | Mortality | | | Lung Lesions | MST, |
|---|---|---|---|---|---|---|
| $NO_2$ | $O_3$ | D/T | % | Change, % | in Survivors | day |
| 0 | 0 | 7/47 | 14.8 | - | 3.00 | 13.6 |
| 6580 | 0 | 23/48 | 47.8* | +33.0 | 3.90* | 12.5* |
| 0 | 980 | 13/48 | 27.0 | +12.2 | 3.15 | 13.3 |
| 6580 | 980 | 6/48 | 12.5 | - 2.3 | 2.69 | 13.6 |

* Significant change from corresponding infected mice exposed to filtered air ($p \leq 0.05$).

## Clearance of Inhaled Bacteria from Lungs

The effects on clearance of viable bacteria from lungs were investigated in mice exposed for 3 hr to mixtures of 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$, or 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$. Groups of 5 mice, either exposed to filtered air or the pollutants were killed at hourly intervals up to 5 hr and at 1,2,3, and 6 days after the respiratory challenge and the concentration of *S. pyogenes* in their lungs was assayed. The clearance rate of viable bacteria from the lungs was markedly delayed after exposure to the 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture. The time required to clear 50% of inhaled bacteria in mice exposed to filtered air was approximately 1.35 hr but 2.18 hr in those exposed to the pollutant mixture. No differences were seen in bacterial clearance upon exposure to 3760 $\mu g/m^3$ (2 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ mixture.

The clearance rates over an extended period were calculated as the number of mice out of the total number of mice killed on a given day having viable *S. pyogenes* present in their lungs. The effects of exposure to mixtures of the pollutants were much more pronounced over this 6 day assay period (Table 4). Among the mice exposed to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture, 15/19 (79%) and 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ mixture 16/18 (89%) showed viable *S. pyogenes* in the lungs, whereas among the corresponding controls 7/20 (35%) and 8/20 (40%) were positive. Thus, it appears that the ability to clear inhaled bacteria is markedly impaired by a single 3 hr inhalation of the pollutant mixtures.

TABLE 4. RETENTION OF INHALED VIABLE *STREPTOCOCCUS* IN LUNGS OF MICE EXPOSED FOR 3 HR TO $NO_2$ AND $O_3$ MIXTURES

| Day of Lung Assay | Concentration, $\mu g/m^3$ 6580/$NO_2$+196/$O_3$ Positive/Total* | | 3760/$NO_2$+98/$O_3$ Positive/Total* | |
|---|---|---|---|---|
| | Control | Expt | Control | Expt |
| 1 | 2/5 | 5/5 | 0/5 | 4/4 |
| 2 | 3/5 | 3/5 | 2/5 | 4/5 |
| 3 | 0/5 | 3/4 | 4/5 | 3/4 |
| 6 | 2/5 | 4/5 | 2/5 | 5/5 |

* Number of mice showing viable *S. pyogenes* out of total number of mice assayed.

SUMMARY

A single 3 hr exposure to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ or 196 $\mu g/m^3$ (0.1 ppm) $O_3$ increased the susceptibility of mice to streptococcal pneumonia. This was demonstrated by excess deaths and reduced survival time compared to infected mice exposed to filtered air. Within the $NO_2$ concentration range of 2820 (1.5 ppm) to 9400 $\mu g/m^3$ (5.0 ppm) and $O_3$ ranging from 98 (0.05 ppm) to 980 $\mu g/m^3$ (0.5 ppm) the response was linear whereby an increase in concentration of the pollutant resulted in increased excess mortalities.

The effect of a single 3 hr exposure to the $NO_2$ and $O_3$ mixtures was additive. At most concentrations the excess mortality was equivalent to the sum of excess deaths resulting from exposure to each individual pollutant. At the higher concentrations of the pollutant mixture (i.e., 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 980 $\mu g/m^3$ (0.5 ppm) $O_3$) a 1 hr exposure was equally effective in inducing excess mortality as was the 3 hr exposure. However, at the lower concentrations of the pollutants the 3 hr exposure was more effective.

The ability to clear inhaled bacteria from lungs was impaired by the exposure to the $NO_2$ and $O_3$ mixture. A marked increase in the time required to clear 50% of viable bacteria within 4 to 5 hr after the infectious challenge was seen upon exposure to 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture. Moreover, a large percentage of mice had *S. pyogenes* present in their lungs 6 days after the respiratory challenge.

A 3 hr exposure to either 6580 $\mu g/m^3$ (3.5 ppm) $NO_2$ or 980 $\mu g/m^3$ (0.5 ppm) $O_3$ but not to a mixture of the two markedly enhanced the susceptibility of mice to influenza infection. Exposure to these pollutants within 24 hr after a respiratory challenge with influenza virus resulted in excess mortality, reduced survival time and increase in lung consolidation.

## MULTIPLE 3-HR EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES

### Mortality and Survival Time

In studies of the effects of multiple exposures, mice were exposed daily for 3 hr, 5 days/week, for 1, 2 and 4 weeks to mixtures consisting of 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.5 ppm) $O_3$, or 2820 $\mu g/m^3$ (1.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$. Within 1 hr after termination of the final exposure the mice, along with control mice exposed to filtered air, were challenged with *S. pyogenes* aerosol. The excess mortalities, based on exposure of 48 mice to the 2820 $\mu g/m^3$ (1.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture, and 104 mice to the 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ mixture are shown in Figure 2.

Repeated daily exposure to the mixture consisting of 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ followed by the infectious challenge resulted in significant excess deaths over those observed in control mice. The excess mortalities were present irrespective of the number of the daily 3-hr exposures.

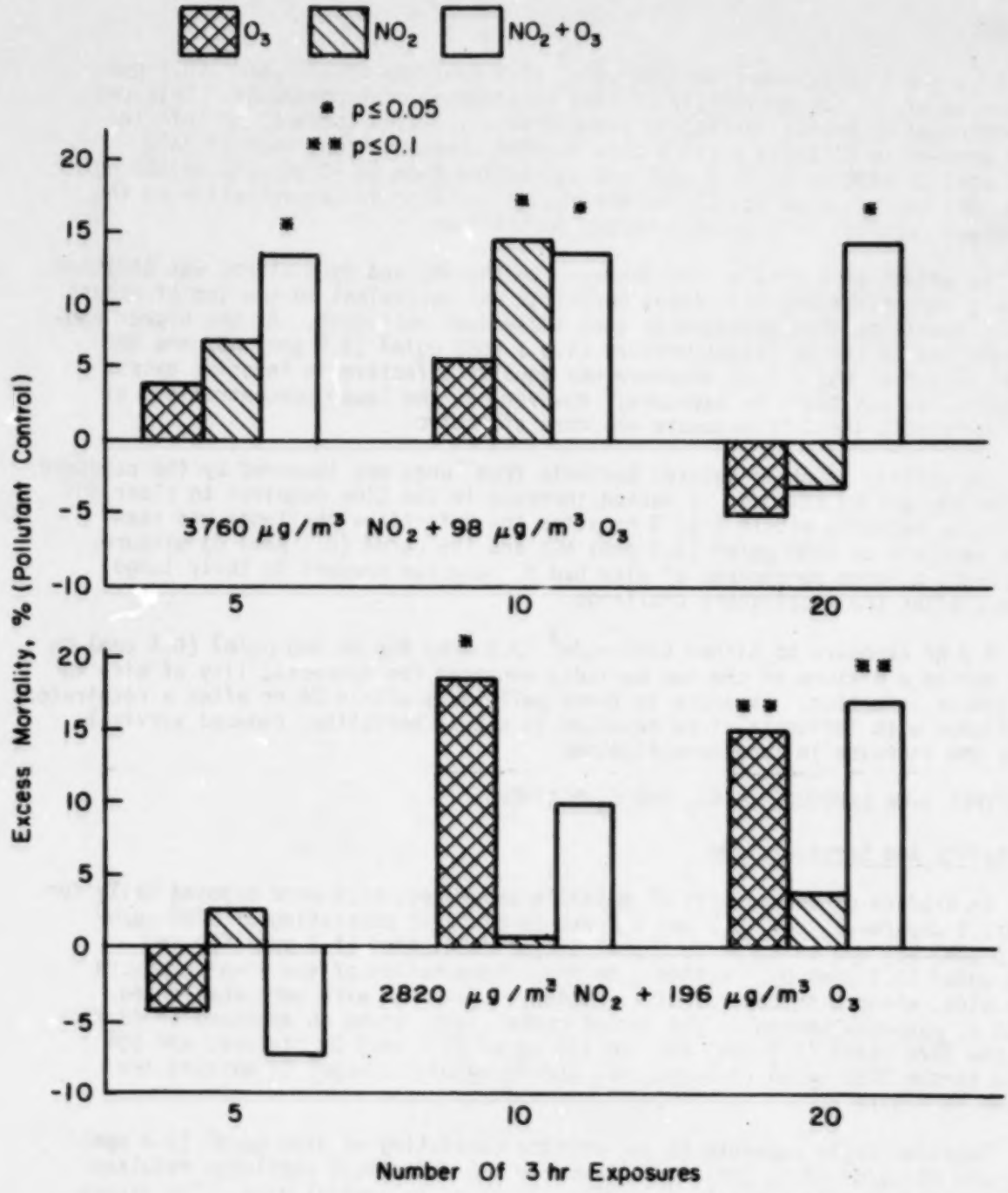


Figure 2. Excess mortality in mice after multiple daily 3 hr exposures to $NO_2$ and $O_3$ mixtures and respiratory challenge with Streptococcus aerosol.

Among the mice exposed to the individual pollutants, only those exposed 10 times to 3760 μg/m$^3$ $NO_2$ showed significant excess mortality when compared to infected mice exposed to filtered air. Thus, daily 3-hr exposure to the individual pollutants had no major effect on the mortality rates. On the other hand, daily exposures to the $NO_2$ and $O_3$ mixture containing the same concentration of each pollutant resulted in significant excess mortality. This suggests a synergistic relation between the two pollutants that makes them more effective in reducing resistance to respiratory infection. At the same time the absence of effect of $NO_2$ after 20 daily exposure could suggest a possible development of tolerance to this pollutant.

Results of daily 3-hr exposures to a mixture consisting of 2820 μg/m$^3$ (1.5 ppm) $NO_2$ and 196 μg/m$^3$ (0.1 ppm) $O_3$ indicate that there were no remarkable differences in mortalities at any of the exposure conditions after five daily exposures. However, marked excess deaths were present after 10 or 20 exposures to $O_3$ or the $NO_2$ and $O_3$ mixture. The fact that excess mortalities seen upon inhalation of the mixture approximated those observed in mice exposed to $O_3$ only, suggests that they were due to the presence of $O_3$ in the mixture.

In a single experiment the effects of daily 3-hr exposures 5 days/week for 4 weeks to 3760 μg/m$^3$ (2.0 ppm) $NO_2$ and 98 μg/m$^3$ (0.05 ppm) $O_3$ mixtures on the resistance to influenza infection were studied. The mice were exposed to the pollutants and within 1 hr after termination of the final exposure were challenged with $A_2$/Taiwan influenza virus aerosol. The mortality and survival rates and lung consolidation seen in Table 5 indicate that inhalation of these pollutants had no pronounced effect on the resistance to influenza.

TABLE 5. EFFECT OF DAILY 3 HR EXPOSURE, 5 DAYS/WEEK FOR 4 WEEKS TO $NO_2$ AND $O_3$ MIXTURES ON RESISTANCE TO INFLUENZA INFECTION

| Concn, μg/m$^3$ | | Mortality | | | Lung | MST, |
|---|---|---|---|---|---|---|
| $NO_2$ | $O_3$ | D/T | % | Change, % | Lesions | day |
| 0 | 0 | 18/48 | 37.5 | - | 3.21 | 16.5 |
| 3760 | 0 | 23/48 | 47.9 | +10.4 | 3.42 | 16.2 |
| 0 | 98 | 18/48 | 37.5 | 0 | 2.94 | 16.8 |
| 3760 | 98 | 21/48 | 43.8 | + 6.3 | 3.15 | 16.2 |

Immune Response

Mice were exposed 3 hr/day, 5 days/week, for 4 weeks to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$, 98 $\mu g/m^3$ (0.05 ppm) $O_3$, or to a mixture containing the same concentrations of the two pollutants. Within 1 hr after termination of the final exposure to the pollutants, the mice were vaccinated with 0.1 ml of chick embryo $A_2$/Taiwan/1/64 influenza vaccine (Lot No. B80549 Zonomune, Eli Lilly and Company, Indianapolis, IN). A single subcutaneous injection of approximately 300 CCA units of the vaccine was used. Control mice received subcutaneous injection of phosphate-buffered saline. After the vaccination all mice were kept in isolated animal room in clean ambient air.

At 2, 12 and 22 weeks after the vaccination groups of mice were exsanguinated and blood serum was collected to determine the hemagglutination-inhibition (HI) antibody levels. The primary HI antibody titer in all groups of mice was <8. At the same time intervals (2, 12 and 22 weeks) groups of mice representing all exposures to the pollutants were challenged by the respiratory route with infectious $A_2$/Taiwan influenza virus and mortality and survival time were recorded. The surviving mice were exsanguinated 16 days after the infectious challenge to determine the secondary HI antibody formation and lung consolidation.

Because of the inadvertent use of low challenge dose and the ensuing low mortality among the nonvaccinated mice it was difficult to determine the protection provided by the vaccine at 2 and 12 weeks after the vaccination. The mortality among the nonvaccinated mice was 12.5% (3/24) and 8.3% (2/24) whereas that among vaccinated mice exposed to filtered air was 0% (0/24) and 4.2% (1/24). The mortality rates in mice exposed to the pollutants ranged from 0% (0/24) to 8.3% (2/24). At 22 weeks the vaccine afforded significant protection against the infectious challenge. Mortality in nonvaccinated mice was 31.6% (12/38), in vaccinated mice exposed to filtered air 5.7% (2/35) and in those exposed to the pollutants ranged from 6.1 to 12.8%. Thus the mortality of mice vaccinated with influenza virus vaccine and challenged with infectious virus was not affected by exposure to the pollutants.

The lung consolidation, HI titers and percent seroconversion in surviving mice exposed to the pollutants for 4 weeks, vaccinated and challenged with infectious influenza virus at various time periods after the vaccination are shown in Table 6. At 4 weeks after vaccination, mice exposed to either 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$, 98 $\mu g/m^3$ (0.05 ppm) $O_3$ or to the mixture consisting of the two pollutants, showed significant increase in lung consolidation compared to the vaccinated mice exposed to filtered air. At 14 weeks after vaccination only mice exposed to $NO_2$ showed significantly higher lung lesion scores than vaccinated mice held in filtered air. At 24 weeks more severe lung consolidation were seen in all mice exposed to the pollutants, but the statistical significance of the differences could be ascertained only for the mice exposed to 98 $\mu g/m^3$ (0.05 ppm) $O_3$. The lung lesion scores in nonvaccinated mice challenged with infectious virus were 2.61, 2.50, and 2.57 at 4, 14 and 24 weeks, respectively.

TABLE 6. LUNG CONSOLIDATION AND GEOMETRIC MEAN RECIPROCAL HI ANTIBODY TITERS IN MICE EXPOSED 3 HR/DAY, 5 DAYS/WEEK FOR 4 WEEKS TO $NO_2$ AND $O_3$ MIXTURES, VACCINATED AND CHALLENGED WITH INFECTIOUS INFLUENZA VIRUS AEROSOL

| Concn., $\mu g/m^3$ | | Week after Vaccination | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 | | | | 14 | | | | 24 | | | |
| | | Lung Consol | | HI | Seroconv | Lung Consol | | HI | Seroconv | Lung Consol | | HI | Seroconv |
| $NO_2$ | $O_3$ | Mean | SE | Titer | % | Mean | SE | Titer | % | Mean | SE | Titer | % |
| 0 | 0 | 0.48 | 0.12 | 19.5 | 78 | 0.88 | 0.20 | 12.9 | 42 | 1.06 | 0.20 | 34.7 | 67 |
| 3760 | 0 | 0.92* | 0.15 | 9.1* | 44 | 1.96* | 0.29 | 49.0* | 85 | 1.31 | 0.25 | 36.3 | 71 |
| 0 | 98 | 1.00* | 0.22 | 7.6* | 20 | 0.79 | 0.12 | 19.5 | 75 | 1.79* | 0.27 | 93.3 | 86 |
| 3760 | 98 | 1.05* | 0.20 | 20.9 | 63 | 1.00 | 0.17 | 30.2 | 75 | 1.24 | 0.21 | 72.4 | 79 |

* Significant change from corresponding infected mice exposed to filtered air ($p \leq 0.05$).

At 4 weeks after vaccination, exposure to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ or to 98 $\mu g/m^3$ (0.05 ppm) $O_3$ caused a significant depression of the secondary HI antibody response (Table 6). At 14 weeks a 4-fold increase in HI antibody titer was seen in mice exposed to $NO_2$ and a 2-fold increase in those exposed to the $NO_2$ and $O_3$ mixture. This was in agreement with the lung consolidation data, suggesting extensive virus replication. At the same time period, marked increase in HI seroconversion (i.e., the percentage of mice showing titers of $\geq 8$) was seen in all groups of mice exposed to the individual or mixture of the pollutants. The generally higher HI antibody titers seen in all mice at 24 weeks reflect the higher infectious virus dose used in the challenge. Exposure to 98 $\mu g/m^3$ (0.5 ppm) $O_3$ or the $NO_2$ and $O_3$ mixture resulted in an approximately 3- and 2-fold increase of HI antibody titers. The higher HI antibody titer was consistent with pulmonary consolidation especially in mice exposed to 98 $\mu g/m^3$ (0.05 ppm) $O_3$.

## SUMMARY

Daily 3 hr exposures, 5 days/week for 1, 2 or 4 weeks to mixtures consisting of 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ resulted in significant excess mortalities from streptococcal pneumonia. Inhalation of the individual pollutants had no effect on the susceptibility to infection, thus suggesting a possible synergistic interaction upon exposure to the pollutant mixture. An identical exposure regimen to 2820 $\mu g/m^3$ (1.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ mixture induced significant excess mortalities only after 4 week exposure.

Susceptibility to influenza infection was not affected by the multiple exposure to either of the pollutants or pollutant mixture.

The daily 3 hr exposure, 5 days/week for 4 weeks to 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 98 $\mu g/m^3$ (0.05 ppm) $O_3$ did not diminish the effectiveness of influenza vaccine in protecting mice from a respiratory challenge with infectious influenza virus. However, vaccinated mice exposed to the pollutants in general had more severe lung lesions, increased HI antibody titers and increased rate of seroconversion.

## LONG-TERM EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES

Studies were conducted to determine the effect of long-term exposure to various concentrations of $NO_2$, $O_3$ and $NO_2$ and $O_3$ mixtures on the resistance to streptococcal pneumonia as measured by mortality and survival rates, clearance of inhaled bacteria from the lungs and phagocytic activity of alveolar macrophages. In addition, body weights, lung edema and changes in various hematological and clinical pathology parameters were determined. Selected tissues from the respiratory tract were examined by conventional and scanning electron microscopy. The exposure conditions used in the experiments were:

| Continuous Exposure 24 hr/day, 7 days/week | Peak Exposure 3 hr/day, 5 days/week |
|---|---|
| Filtered Air | Filtered Air |
| Filtered Air | 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ |
| 188 $\mu g/m^3$ (0.1 ppm) $NO_2$ | 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ |
| Filtered Air | 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ |
| 188 $\mu g/m^3$ (0.1 ppm) $NO_2$ | 940 $\mu g/m^3$ (0.5 ppm) $NO_2$ |
| Filtered Air | 196 $\mu g/m^3$ (0.1 ppm) $O_3$ |

Mortality and Survival Time

Mice exposed to the pollutants for 1,2,3 or 6 months were challenged with the *S. pyogenes* aerosol and held in clean air environment for a 14-day observation period. Mortality rates and mean survival time for each exposure condition are shown in Table 7.

The 1 or 2 month exposure did not induce any marked changes in mortality rates. The exception was a significant decrease in mortality among mice exposed for 1 month to filtered air with the superimposed daily 3 hr peaks of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture. In this treatment group the mortality was 14.0% below and the survival time 1.3 day above that of infected mice exposed to filtered air.

The 3 month exposure resulted in significant changes in mortality rates. Excess mortality was seen in mice exposed to filtered air with superimposed $NO_2$ and $O_3$ peaks (+21.0%) and those exposed to filtered air with superimposed daily 3 hr peak of 940 $\mu g/m^3$ $NO_2$ (+11.2%). The corresponding mean survival time decreased by 2.5 and 1.8 days. On the other hand, significant decrease in mortality was seen among mice continuously exposed to 188 $\mu g/m^3$ $NO_2$ with daily 3 hr peaks of $NO_2$ and $O_3$ mixture (-15.3%) or $NO_2$ (-17.0%). The decrease was not accompanied by significant changes in mean survival time, however.

The 6 month exposure resulted in excess mortalities in all treatment groups. The significance of the differences in mortalities could be ascertained for the group of mice exposed to filtered air with the $NO_2$ and $O_3$ peaks (+27.2%) and those continuously exposed to 188 $\mu g/m^3$ $NO_2$ with the superimposed $NO_2$ and $O_3$ peaks (+14.8%). The inability to demonstrate the statistical significance of the excess mortalities in the other two $NO_2$ treatment groups can be ascribed to the small number of mice used in the experiments.

TABLE 7. EFFECT OF EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES ON MORTALITY AND SURVIVAL TIME OF MICE CHALLENGED WITH *STREPTOCOCCUS* AEROSOL

| Exposure Concn., $\mu g/m^3$ | | | Duration of Exposure, Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 hr/day | 3 hr/day | | 1 | | | 2 | | | 3 | | | 6 | | |
| | | | Mortality | | MST | Mortality | | MST | Mortality | | MST | Mortality | | MST |
| $NO_2$ | $NO_2$ | $O_3$ | D/T | % | day | D/T | % | day | D/T | % | day | D/T | % | day |
| 0 | 0 | 0 | 24/126 | 19.1 | 12.3 | 21/87 | 24.1 | 12.2 | 62/156 | 39.7 | 11.3 | 9/44 | 20.5 | 12.8 |
| 0 | 940 | 196 | 5/99 | 5.1* | 13.6* | 20/72 | 27.8 | 12.0 | 94/155 | 60.7* | 8.8* | 21/44 | 47.7* | 11.0* |
| 188 | 940 | 196 | 21/127 | 16.5 | 12.5 | 14/80 | 17.5 | 12.9 | 38/156 | 24.4* | 11.9 | 18/51 | 35.3** | 11.2* |
| 0 | 940 | 0 | 28/127 | 22.1 | 11.7 | 26/196 | 27.1 | 12.0 | 86/169 | 50.9* | 9.5* | 13/46 | 28.3 | 11.8* |
| 188 | 940 | 0 | 18/127 | 14.2 | 12.7 | 28/117 | 23.9 | 12.1 | 39/172 | 22.7* | 12.4 | 14/42 | 33.3 | 11.5* |
| 0 | 0 | 196 | 19/127 | 15.0 | 12.9 | 26/107 | 24.3 | 12.0 | 70/158 | 44.3 | 11.1 | 12/47 | 25.5 | 12.3 |

Significant change from corresponding infected mice exposed to filtered air.

* $p \leq 0.05$.

** $p < 0.01$

The differences in percent mortality for the various exposure groups are shown in Figure 3. The data indicate that the mode of exposure to the pollutants is important in altering the host defense against respiratory infection. Most effective in reducing the resistance to streptococcal pneumonia appeared to be the daily 3 hr exposure to 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture in mice otherwise kept in filtered air. During the first month of exposure there was an increase in resistance to infection. This was followed by a gradual decrease in resistance demonstrated by significant excess mortalities after the 3 and 6 month exposures.

A gradual increase in resistance to the respiratory infection was seen during the first 3 months of continuous exposure to 188 $\mu g/m^3$ $NO_2$ with the superimposed daily 3 hr peak of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture or upon continuous exposure to 188 $\mu g/m^3$ $NO_2$ with 940 $\mu g/m^3$ $NO_2$ peak. However, after 6 month exposure the effect was reversed and marked increase in susceptibility to the respiratory infection was observed. Daily 3 hr exposure to 940 $\mu g/m^3$ $NO_2$ also resulted in increased susceptibility to the infection after 3 and 6 month. However, the daily 3 hr exposure to 196$\mu g/m^3$ $O_3$ for up to 6 months had no effect on the susceptibility to the streptococcal pneumonia.

The effect of inhalation of the pollutants before and after the infectious challenge was investigated in conjunction with the 1, 2 and 3 month exposures. In these studies, instead of being maintained in filtered air for the 14 day observation period after the infectious challenge, mice were kept in the same environment to which they were exposed before the challenge. The results summarized in Table 8 indicate that inhalation of the pollutants during the 14 day post-infection period had a pronounced effect on mortality rates and survival time. After 1 month exposure the changes were small and not significant. Nevertheless, in contrast to mice maintained in filtered air after the infectious challenge, all exposures resulted in excess mortalities and slight decrease in the survival time. After 2 or 3 months exposure, when the mice were held for 14 days in polluted atmosphere after the challenge with *S. pyogenes* aerosol, marked excess mortality and decreased survival time was seen.

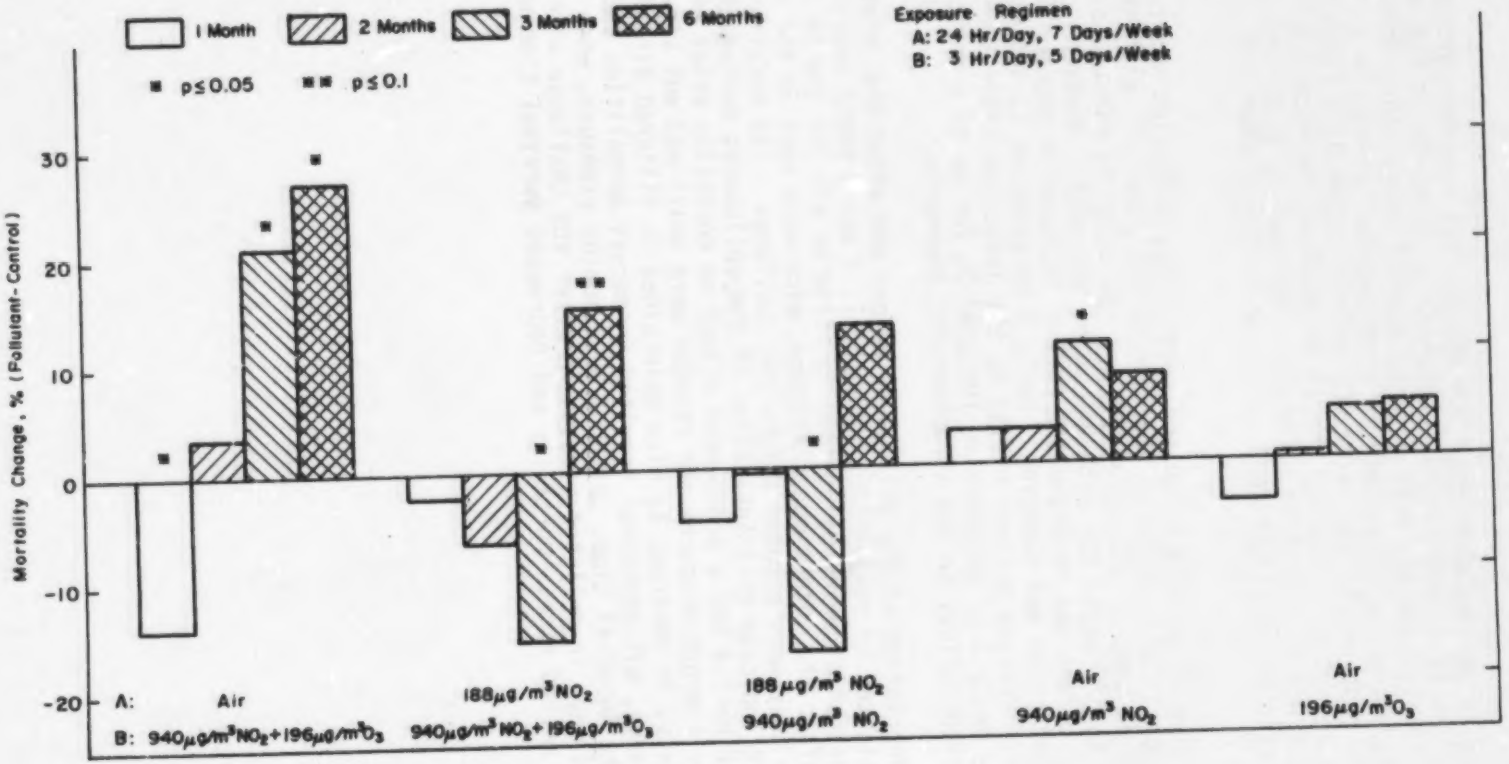


Figure 3. Changes in mortality from streptococcal pneumonia after various exposure regimens to $NO_2$ and $O_3$ mixtures.

TABLE 8: CHANGES IN MORTALITY AND SURVIVAL RATES OF MICE EXPOSED TO $NO_2$ AN $O_3$ MIXTURES, CHALLENGED WITH *STREPTOCOCCUS* AEROSOL AND MAINTAINED FOR 14 DAYS EITHER IN FILTERED AIR OR POLLUTANT MIXTURES

| Exposure Concn., µg/m³ | | | Exposure Condition | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P → IC → A[a] | | | | | P → IC → P[a] | | | | |
| | | | Mortality | | | Survival Time | | Mortality | | | Survival Time | |
| 24 hr/day | 3 hr/day | | | | | | | | | | | |
| $NO_2$ | $NO_2$ | $O_3$ | D/T | % | Change | Day | Change | D/T | % | Change | Day | Change |
| 1 month | | | | | | | | | | | | |
| 0 | 0 | 0 | 24/126 | 19.1 | - | 12.3 | - | 0/78 | 0 | - | 14.0 | - |
| 0 | 940 | 196 | 5/99 | 5.1 | -14.0* | 13.6 | + 1.3 | 2/78 | 2.6 | + 2.6 | 13.9 | - 0.1 |
| 188 | 940 | 196 | 21/127 | 16.5 | - 2.6 | 12.5 | + 0.2 | 3/77 | 3.9 | + 3.9* | 13.8 | - 0.2 |
| 0 | 940 | 0 | 28/127 | 22.1 | + 3.0 | 11.7 | - 0.6 | 2/78 | 2.6 | + 2.6 | 13.8 | - 0.2 |
| 188 | 940 | 0 | 18/127 | 14.2 | - 4.9 | 12.7 | + 0.4 | 1/84 | 1.2 | + 1.2 | 13.9 | - 0.1 |
| 2 month | | | | | | | | | | | | |
| 0 | 0 | 0 | 21/87 | 24.1 | - | 12.2 | - | 5/50 | 10.0 | - | 13.2 | - |
| 0 | 940 | 196 | 20/72 | 27.8 | + 3.7 | 12.0 | - 0.2 | 18/52 | 34.6 | +24.6* | 12.0 | - 1.2 |
| 188 | 940 | 196 | 14/80 | 17.5 | - 6.6 | 12.9 | + 0.7 | 11/45 | 24.4 | +14.4** | 12.1 | - 1.1 |
| 0 | 940 | 0 | 26/96 | 27.1 | + 3.0 | 12.0 | - 0.2 | 12/52 | 23.1 | +13.1** | 12.5 | - 0.7 |
| 188 | 940 | 0 | 28/117 | 23.9 | - 0.2 | 12.1 | - 0.1 | 14/67 | 20.9 | +10.9** | 12.5 | - 0.7 |
| 3 month | | | | | | | | | | | | |
| 0 | 0 | 0 | 62/156 | 39.7 | - | 11.3 | - | 4/60 | 6.7 | - | 13.4 | - |
| 0 | 940 | 196 | 94/155 | 60.7 | +21.0* | 8.8 | - 2.5 | 24/60 | 40.0 | +33.3* | 10.8 | - 2.6 |
| 188 | 940 | 196 | 38/156 | 24.4 | -15.3* | 11.9 | + 0.6 | 16/60 | 26.7 | +20.0* | 11.9 | - 1.5 |
| 0 | 940 | 0 | 86/169 | 50.9 | +11.2* | 9.5 | - 1.8 | 8/60 | 13.3 | + 6.6 | 13.2 | - 0.2 |
| 188 | 940 | 0 | 39/172 | 22.7 | -17.0* | 12.4 | + 1.1 | 9/60 | 15.0 | + 8.3 | 12.7 | - 0.7 |

[a] Pollutant → Infectious Challenge → Filtered Air (from Table 7).

[b] Pollutant → Infectious Challenge → Pollutant

Significant change from corresponding infected mice exposed to filtered air.

* $p \leq 0.05$.

** $p \leq 0.1$.

## Clearance of Bacteria from Lungs

To study the effect of $NO_2$ and $O_3$ mixture on the ability to clear viable inhaled bacteria from the lungs, mice were challenged with *S. pyogenes* aerosol after 1, 2 and 3 months exposure to the pollutants. Groups of four mice from each treatment group were killed immediately after and at 1,2,3 and 4 hours after the infectious challenge. Clearance rates of bacteria from lungs of mice exposed to the various treatments are shown in Table 9. Daily 3-hr exposure to a mixture of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ and the continuous exposure to 188 $\mu g/m^3$ $NO_2$ with superimposed 3-hr daily peaks of either the $NO_2$ and $O_3$ mixture or 940 $\mu g/m^3$ $NO_2$ were most effective in delaying the clearance of inhaled bacteria. The other two exposure conditions, namely $NO_2$ and $O_3$ peaks only, were not effective and in some instances the clearance rate of bacteria from the lungs was faster than that in the control mice.

TABLE 9. CLEARANCE OF INHALED VIABLE *STREPTOCOCCUS* FROM LUNGS OF MICE EXPOSED TO $NO_2$ AND $O_3$ MIXTURES

| Exposure Concn., $\mu g/m^3$ | | | Estimated $t_{50}$ Rate[a] | | |
|---|---|---|---|---|---|
| 24 hr/day | 3 hr/day | | Duration of Exposure (Month) | | |
| $NO_2$ | $NO_2$ | $O_3$ | 1 | 2 | 3 |
| 0 | 0 | 0 | 1.34 | 1.03 | 1.36 |
| 0 | 940 | 196 | 1.57 | 1.26 | 1.88 |
| 188 | 940 | 196 | 1.46 | 1.11 | 1.63 |
| 0 | 940 | 0 | 1.10 | 1.00 | 1.28 |
| 188 | 940 | 0 | 1.59 | 1.35 | 1.82 |
| 0 | 0 | 196 | 0.90 | 1.30 | 1.19 |

[a] Time in hours required to clear 50% of inhaled bacteria from lungs.

Pulmonary Cellular Defense

Limited experiments were conducted to determine the effect of 1,2, and 3 month exposure to the pollutants on alveolar macrophages. The treatment groups included in these studies were control mice, those continuously exposed to 188 $\mu g/m^3$ $NO_2$ with 3 hr peaks of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture, and mice exposed to daily peaks of either $NO_2$ and $O_3$ mixture or 940 $\mu g/m^3$ $NO_2$. After each exposure, groups of 5 to 7 mice were killed and their lungs lavaged. The total cell count, the percentage of alveolar macrophages and their viability and phagocytic activity were determined in the lavage fluid (Table 10).

The total cell count, which contained 96.7 to 99.3% alveolar macrophages, appeared to be influenced by the pollutants, although there was no apparent relationship to the duration of the exposure. After 1 month all three treatment groups showed a total cell count which was lower than that of the controls and the decrease was significant for mice continuously exposed to 188 $\mu g/m^3$ $NO_2$ with the $NO_2$ and $O_3$ mixture peaks and those exposed to the 3 hr daily peaks of 940 $\mu g/m^3$ $NO_2$. The 2 month exposure did not have any effect on the total cell count. At this exposure period mice exposed daily to peaks of $NO_2$ and $O_3$ mixture showed statistically significant decrease in the percentage of alveolar macrophages, this difference, however, is not considered to be biologically significant. After 3 month exposure reduced total cell counts were seen in mice exposed to the peaks of $NO_2$ and $O_3$ mixture and somewhat reduced counts in those exposed continuously to 188 $\mu g/m^3$ with daily $NO_2$ and $O_3$ mixture peaks.

The viability of the alveolar macrophages was not affected by a 1 or 2 months exposure to the pollutants. However, after 3 month a significant decrease in viability was seen in macrophages isolated from mice exposed to the $NO_2$ and $O_3$ peaks. Some decrease in viability was also seen in macrophages of mice exposed continuously to 188 $\mu g/m^3$ $NO_2$ with the superimposed daily peaks of $NO_2$ and $O_3$ mixture.

The phagocytic activity of alveolar macrophages, expressed as the percent of macrophages which engulf at least one latex sphere after 1 hr incubation at 37°C, was not affected by 1 month exposure to the pollutants. After 2 and 3 months, however, a marked decrease was seen in activity of macrophages from mice exposed to the $NO_2$ and $O_3$ mixture peaks and those continuously exposed to 188 $\mu g/m^3$ $NO_2$ and the superimposed $NO_2$ and $O_3$ peaks. After 2 months reduced phagocytosis was also seen in mice exposed to the 3 hr daily peaks of 940 $\mu g/m^3$ $NO_2$.

TABLE 10. EFFECT OF EXPOSURE TO $NO_2$ AND $O_3$ MIXTURES ON ALVEOLAR MACROPHAGES

| Exposure Concn., $\mu g/m^3$ | | | Cell Count | | | | Macrophage | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 hr/day | 3 hr/day | | Total x$10^6$ | | Macroph % | | Viability, % | | Phagocyt, %[a] | |
| $NO_2$ | $NO_2$ | $O_3$ | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| **1 month** | | | | | | | | | | |
| 0 | 0 | 0 | 1.68 | 0.21 | 98.8 | 0.8 | 93.2 | 1.6 | 87.2 | 0.6 |
| 0 | 940 | 196 | 1.50 | 0.30 | 99.3 | 0.5 | 92.3 | 1.2 | 87.2 | 0.7 |
| 188 | 940 | 196 | 1.04* | 0.27 | 98.8 | 0.6 | 94.9 | 0.7 | 87.8 | 0.6 |
| 0 | 940 | 0 | 1.02* | 0.10 | 98.6 | 0.2 | 89.6 | 1.7 | 89.5 | 0.7 |
| **2 month** | | | | | | | | | | |
| 0 | 0 | 0 | 1.37 | 0.23 | 98.6 | 0.4 | 93.8 | 1.0 | 89.1 | 0.9 |
| 0 | 940 | 196 | 1.54 | 0.21 | 96.8* | 0.9 | 95.2 | 1.1 | 82.1* | 0.6 |
| 188 | 940 | 196 | 1.53 | 0.17 | 97.5 | 0.7 | 95.0 | 1.1 | 86.5* | 0.8 |
| 0 | 940 | 0 | 1.30 | 0.09 | 97.8 | 0.6 | 93.8 | 1.1 | 84.0* | 1.9 |
| **3 month** | | | | | | | | | | |
| 0 | 0 | 0 | 0.94 | 0.11 | 96.7 | 0.7 | 87.3 | 2.2 | 85.8 | 0.7 |
| 0 | 940 | 196 | 0.60* | 0.04 | 97.4 | 0.6 | 78.9* | 1.1 | 83.0* | 1.2 |
| 188 | 940 | 196 | 0.78 | 0.09 | 97.1 | 0.4 | 84.9 | 2.5 | 83.7 | 1.0 |
| 0 | 940 | 0 | 1.04 | 0.11 | 97.4 | 0.8 | 88.1 | 1.4 | 86.2 | 1.0 |

[a] Percent of alveolar macrophages engulfing one or more latex sphere after 1 hr incubation at 37°C (Ratio Macrophages to Latex - 1:200).

* Significant change from corresponding control mice exposed to filtered air ($p \leq 0.05$).

Clinical Pathology

Limited experiments were conducted to determine the effects of exposure to the pollutants on activity of selected blood serum enzymes. Mice were exsanguinated after 1, 3 and 6 months exposure and serum was assayed for the activity of acid phosphatase (AP), cholinesterase (CHE), lactate dehydrogenase (LDH), serum glutamic oxalic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). In addition, gel electrophoresis was used to separate the isoenzyme forms of LDH. The geometric means, standard error, statistical significance of the differences between the means and the number of mice examined in each assay are shown in Figure 4.

In general elevation of SGPT, SGOT and LDH, and depression of AP and CHE activity were observed. Moreover, a significant increase of the LDH-1 isoenzyme with a corresponding decrease in the other four isoenzymes was observed after 6 month exposure. The continuous exposure to 188 $\mu g/m^3$ $NO_2$ with the superimposed 3 hr daily peaks of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture appeared to be most effective in inducing these changes. Because of the small numbers of mice available for these determinations and the variability of the enzyme levels the statistical significance of the differences could be ascertained in only a limited number of exposure conditions.

The changes in serum enzyme activity were in a close agreement with those reported by Kosmider (unpublished data) and Menzel, et al. (17) for guinea pigs exposed to 940 $\mu g/m^3$ $NO_2$ 8 hr/day, 7 days/week for 4 month. In spite of the differences in animal species (mice vs quinea pigs) and the duration and mode of exposure to the pollutants the trends of the changes closely resembled those seen in mice after 6 month continuous exposure to 188 $\mu g/m$ with superimposed daily 3-hr peaks of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$.

| | Kosmider | Menzel | IITRI |
|---|---|---|---|
| AP | ↓* | ↓* | ↓ |
| LDH | ↑* | | ↑ |
| CHE | ↓* | ↓* | ↓* |
| SGOT | | ↑ | ↑* |

(↑ increase; ↓ decrease)
(* significant change from control values)

Elevation of SGOT, SGPT and LDH is usually seen in many pathologic conditions whereas these enzymes are released as the result of damage to heart, liver, muscle and other tissue such as lung. An increase in levels of these enzymes could be indicative of damage to lung resulting from exposure to air pollutants. Acid phosphatase is present in lung lysosomal fractions capable of tissue digestion. Depressed plasma AP in presence of increase in lung tissue AP levels could suggest continuing elevation of phagocytosis in the lung due to inhalation of the pollutants (17).

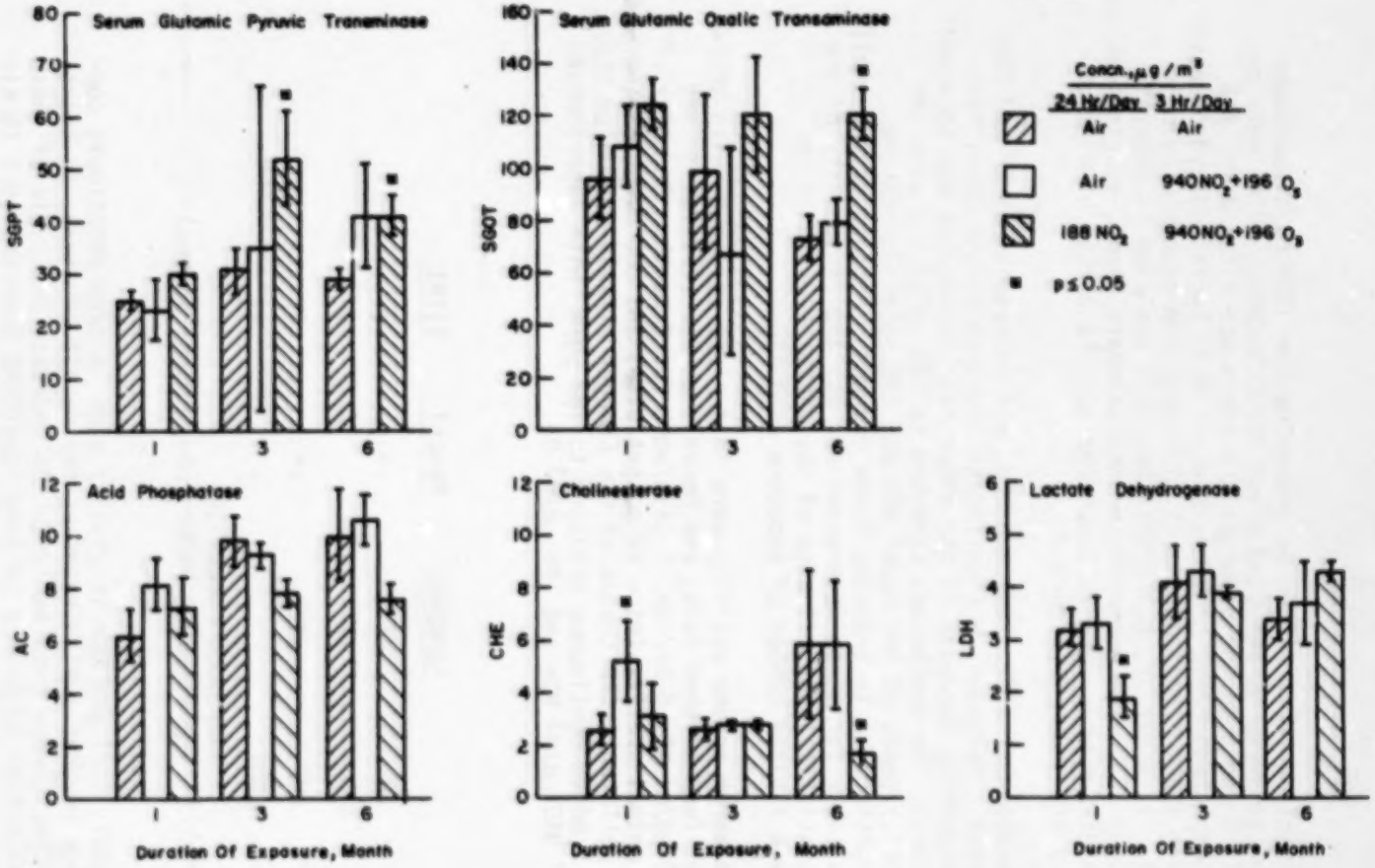


Figure 4. Effect of various regimens of exposure to $NO_2$ and $O_3$ mixtures on blood serum enzymes.

## Clinical Hematology

After 1, 3 and 6 months groups of 4 mice continuously exposed to air or 188 $\mu g/m^3$ $NO_2$ with superimposed 3 hr daily peaks of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture and groups of 4 control mice exposed to air only were exsanguinated and various hematological parameters were determined. Because of the small number of mice used in these exploratory studies it was difficult to ascertain the statistical significance of the observed differences. Moreover, the paucity of information available in the literature on blood characteristics of mice in general and specifically as related to age made the interpretation of the data difficult. Exposure to the pollutants did not have any apparent effect on hemoglobin content or erythrocyte count, whereas the hematocrit values were reduced. White blood cell, reticulocyte, platelet and differential counts showed variations which, however, did not appear to be related to the exposure to the pollutants.

## Pulmonary Edema

The wet-to-dry lung weight ratios expressing the extent of pulmonary edema was determined after exposure to the pollutants. After 1 month the lung water content did not differ between control mice and those exposed to the pollutants. After 3 and 6 month exposure the water content was lower in mice exposed to the pollutants then those exposed to filtered air. The differences, although being only 2%, were statistically significant. One possible explanation for the lower water content in lungs of mice exposed to the pollutants could be the development of tolerance to $O_3$ present in the pollutant mixture.

## Body Weights

Groups of 100 mice selected at random from each exposure treatment were weighed weekly to determine body weight changes which may have occurred as the result of inhalation of the pollutants. In general the weight gains were not affected by the exposure.

## Histopathology

Lung tissues from control mice and those exposed to the pollutant mixtures for 1 and 3 month were examined by conventional and scanning electron microscopy. In addition trachea and nasal cavity tissues were subjected to SEM examination. In general there were no remarkable pathological changes which could be attributed to the exposure to the pollutants.

## SUMMARY

Table 11 represents a summary of the health effects observed upon exposure to the various $NO_2$ and $O_3$ mixture environments. The three experimental treatments shown in the table are daily 3 hr exposure 5 days/week to either 940 $\mu g/m^3$ $NO_2$ or a mixture containing 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$, and continuous exposure to 188 $\mu g/m^3$ $NO_2$ with superimposed daily 3 hr peaks (5 days/week) of 940 $\mu g/m^3$ $NO_2$ and 196 $\mu g/m^3$ $O_3$ mixture. The data represent changes from control mice exposed to filtered air only. All changes of approximately 10% are shown and, as appropriate, the statistical significance of the differences is indicated.

TABLE 11. SUMMARY OF HEALTH EFFECTS IN MICE EXPOSED FOR 6 MONTHS TO $NO_2$ OR $NO_2$ AND $O_3$ MIXTURES

| Exposure Concn., µg/m³ 24 hr/day $NO_2$ | 3 hr/day $NO_2$ | 3 hr/day $O_3$ | Mortality | Survival Time | Lung Clearance | Cell Count | AM Viability | AM Phagocyt. | AP | LDH | CHE | SGPT | SGOT | LDH 1 | LDH 2-5 | Hct | Hemoglobin | RBC | WBC | Platelets | Neutrophils | Lymphocytes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 month | | | | | | | | | | | | | | | | | | | | | | |
| 0 | 940 | 0 | + | - | o | o | o | + | (+) | + | o | o | + | o | o | u | o | o | u | - | u | u |
| 0 | 940 | 196 | (-) | + | - | - | o | o | + | o | (+) | o | + | o | o | (-) | o | o | u | - | u | u |
| 188 | 940 | 196 | - | o | - | (-) | o | + | + | (-) | + | + | + | o | - | u | o | o | u | - | u | u |
| 3 month | | | | | | | | | | | | | | | | | | | | | | |
| 0 | 940 | 0 | (+) | (-) | o | + | o | o | o | - | o | + | + | o | o | u | o | o | u | o | u | u |
| 0 | 940 | 196 | (+) | (-) | - | (-) | (-) | o | o | o | o | + | - | o | o | (-) | o | o | u | o | u | u |
| 188 | 940 | 196 | (-) | (+) | - | - | o | - | - | - | (+) | (+) | + | o | o | o | o | o | u | o | u | u |
| 6 month | | | | | | | | | | | | | | | | | | | | | | |
| 0 | 940 | 0 | + | (-) | n | n | n | n | n | n | n | n | n | n | n | n | n | n | n | n | n | n |
| 0 | 940 | 196 | (+) | (-) | n | n | n | n | n | o | o | + | o | (+) | (-) | - | o | o | + | + | (+) | (-) |
| 188 | 940 | 196 | (+) | (-) | n | n | n | n | - | + | (-) | (+) | (+) | (+) | (-) | (-) | o | o | - | o | o | o |

\+ increase
\- decrease
o no change
( ) significant change from control mice ($p \leq 0.1$)
n not done

The 6 month exposure to any of the pollutant combinations resulted in a marked increase in the susceptibility to streptococcal pneumonia. This was manifested by excess mortality and reduced mean survival time. The increase in susceptibility was much more pronounced after 1, 2 or 3 months when the animals continued to be exposed to the pollutants for 14 days after the infectious challenge. After a 3 month exposure to the $NO_2$ and $O_3$ mixtures there was a marked decrease in the ability of the animals to clear inhaled *S. pyogenes* from their lungs. This impairment, however, was not present in mice exposed daily for 3 hr to 940 $\mu g/m^3$ $NO_2$.

Pulmonary cellular defenses were somewhat affected by the 3 month exposure to the mixture of the pollutants but not by the daily exposure to $NO_2$ only. The total cell count in the fluid lavaged from the lungs was markedly reduced as was the viability and phagocytic activity of the alceolar macrophages.

In non-infected mice the 6 month exposure to 188 $\mu g/m^3$ $NO_2$ with the superimposed daily 3 hr $NO_2$ and $O_3$ mixture peaks resulted in significant decrease in serum cholinesterase, increase in SGPT and SGPT and shift to LDH 1 isoenzyme. In the same treatment group a decrease in hematocrit and an increase in platelet counts was seen. A similar trend in changes was seen in mice daily exposed for 3 hr to the $NO_2$ and $O_3$ mixture.

EXPOSURE TO $SO_2$

The effects of $SO_2$ on the resistance to respiratory infection was studied in a series of experiments. The studies included single and multiple 3-hr exposures as well as continuous exposures for up to 3 months to 13100 $\mu g/m^3$ (5.0 ppm) $SO_2$. Exploratory studies were also conducted to determine the effects of $SO_2$ in high humidity environment (85% RH), and in presence of respirable size carbon particles, zinc sulfate particles, and mixture of $NO_2$ and $O_3$.

Short-term 3-hr Exposure

The mortality and mean survival time resulting from single and multiple 3-hr exposures to $SO_2$ followed by respiratory challenge with *S. pyogenes* aerosol are shown in Table 12. It is apparent from these data that inhalation of 13100 $\mu g/m^3$ (5.0 ppm) $SO_2$ had no effect on the susceptibility to streptococcal pneumonia. Similar lack of effect was seen upon a single 3 hr exposure to $SO_2$ at 85% RH (46.2% vs 39.5% mortality in control mice), in presence of 2200 $\mu g/m^3$ carbon particles (35.6% vs 38.2% mortality in con-rol mice) or in presence of 800 $\mu g/m^3$ of zinc sulfate particles (29.1% mortality in $SO_2$ exposed mice vs 27.1% mortality in mice exposed to $SO_2$ and zinc sulfate mixture). Exposure to 13100 $\mu g/m^3$ $SO_2$ in mixture with 3760 $\mu g/m^3$ (2.0 ppm) $NO_2$ and 196 $\mu g/m^3$ (0.1 ppm) $O_3$ resulted in a significant excess mortality (26.3%) which, however, was probably due to the presence of $NO_2$ and $O_3$ in the mixture.

Thus it appears that exposure to 13100 $\mu g/m^3$ (5.0 ppm) $SO_2$ alone or in mixture with other pollutants has no effect on the susceptibility to streptococcal pneumonia.

TABLE 12. MORTALITY AND SURVIVAL TIME OF MICE DAILY EXPOSED FOR 3 HR TO 13100 $\mu g/m^3$ $SO_2$ AND CHALLENGED WITH *STREPTOCOCCUS* AEROSOL

| Number of Daily 3-hr Exposures | Filtered Air | | | 13100 $\mu g/m^3$ $SO_2$ | | | |
|---|---|---|---|---|---|---|---|
| | Mortality | | MST | Mortality | | | MST |
| | D/T | % | day | D/T | % | Change (%) | day |
| 1 | 90/373 | 24.1 | 12.1 | 97/372 | 26.1 | +2.0 | 12.0 |
| 5 | 13/48 | 27.1 | 11.6 | 13/48 | 27.1 | 0 | 12.0 |
| 10 | 19/48 | 39.6 | 10.4 | 18/47 | 38.3 | -1.3 | 10.5 |
| 15 | 15/47 | 31.9 | 11.5 | 14/46 | 30.4 | -1.5 | 11.5 |

Continuous Exposure

In studies of effects of continuous exposures to 13100 $\mu g/m^3$ (5.0 ppm) $SO_2$ for up to 3 months various physiological response parameters were measured. They included resistance to streptococcal and viral pneumonias, clearance of inhaled bacteria from the lungs, phagocytic activity of alveolar macrophages, lung edema, hematology, clinical pathology and conventional and scanning electron microscopic examination of the respiratory tract tissue.

After 1, 2, or 3 months of continuous exposure to $SO_2$, mice were challenged by the respiratory route with either *S. pyogenes* or influenza virus aerosols. The summary shown in Table 13 indicate that in general the $SO_2$ exposure had only a slight effect on resistance to streptococcal pneumonia. A significant excess mortality was seen after 2 month but not after 1 or 3 month exposure. In mice challenged with influenza virus mortality rates were significantly higher after 1 month, but significantly lower after 3 month exposure to $SO_2$. It was interesting to note that lung lesions in mice surviving the influenza infection were consistently higher in those exposed to $SO_2$ than control mice exposed to filtered air, suggesting that the infection was more severe in mice exposed to $SO_2$.

After 1 and 2 months exposure to $SO_2$, viable inhaled *S. pyogenes* was cleared slightly faster from lungs of mice exposed to $SO_2$ then from control mice exposed to filtered air. The $t_{50}$ in mice exposed for either 1 or 2 months to $SO_2$ was 1.05 hr while for corresponding control mice were 1.40 and 1.27 hr. After 3 months the $t_{50}$ for mice exposed to $SO_2$ was 1.08 hr and for the corresponding control mice 0.70 hr. Inasmuch as the 1.08 hr value was in close agreement with the $t_{50}$ values seen after 1 and 2 months $SO_2$ exposure, it is assumed that the $t_{50}$ obtained in control mice is in error. Thus, it can be concluded that exposure to $SO_2$ had no effect on clearance rate of inhaled bacteria from the lungs.

TABLE 13. MORTALITY AND MEAN SURVIVAL TIME OF MICE EXPOSED TO 13100 $\mu g/m^3$ $SO_2$ AND CHALLENGED WITH *STREPTOCOCCUS* OR INFLUENZA VIRUS AEROSOL

| Duration of $SO_2$ Exposure, Month | Mortality | | | | | Survival Time, day | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | $SO_2$ | | Change | Control | $SO_2$ | Change |
| | D/T | % | D/T | % | % | | | % |
| *Streptococcus* | | | | | | | | |
| 1 | 13/131 | 9.9 | 10/129 | 7.8 | - 2.1 | 13.4 | 13.5 | +0.1 |
| 2 | 6/120 | 5.0 | 15/120 | 12.5 | + 7.5* | 13.7 | 13.1 | -0.6 |
| 3 | 4/48 | 8.3 | 5/48 | 10.4 | + 2.1 | 13.4 | 13.3 | -0.1 |
| Influenza Virus | | | | | | | | |
| 1 | 35/50 | 70.0 | 48/55 | 87.3 | +17.3* | 11.4 | 11.3 | -0.1 |
| 2 | 23/48 | 47.9 | 30/48 | 62.5 | +14.6 | 12.0 | 11.5 | -0.5 |
| 3 | 33/48 | 68.8 | 23/48 | 47.9 | -20.9* | 9.7 | 12.1 | +2.4 |

* Significant change from corresponding infected mice exposed to filtered air ($p \leq 0.05$).

Examination of fluid lavaged from lungs of mice exposed to $SO_2$ did not demonstrate any significant changes in total and differential cell counts, nor the percent viability of alveolar macrophages or their ability to phagocytize polystyrene latex spheres.

The wet-to-dry lung weight ratios examined after 1, 2 and 3 month exposure to $SO_2$ showed no significant differences.

During the first month of exposure an increase in body weight was seen in mice exposed to $SO_2$ as well as those exposed to filtered air. Thereafter the weights leveled off without showing any remarkable differences between the control and experimental animals during the remaining exposure period.

Rectal temperatures of randomly selected mice from the exposure and control group were determined during the 3 month period. Temperatures decreased somewhat in both groups after 1 week and remained depressed for 2 to 3 weeks, at which time, recovery to normal body temperatures followed. Irrespective of the exposure treatment marked decrease in body temperature was seen during the first 24 hr after the infectious challenge with *S. pyogenes*, and a 3°C decrease in temperature was seen in mice which died due to the streptococcal pneumonia.

For clinical pathology and hematologic examination blood samples were obtained from orbital bleeding using heparinized micro-hematocrit capillary tubes. The hematological assay included hematocrit hemoglobin, red, white and differential blood cell counts, reticulocytes, platelets and sedimentation rate. Clinical pathology included activity in serum of alkaline phosphatase, lactic dehydrogenase (LDH) and LDH isoenzymes, αhydroxybutyrate alkaline dehydrogenase (HBDH) and isocitric dehydrogenase (ICDH). The variations observed in the various measurements were within the expected normal range and were not related to the exposure.

Lungs, trachea, and nasal cavities from mice exposed for 1, 2, and 3 months to $SO_2$ were examined by scanning electron microscopy. Thickened alveolar septa and enlarged pores were seen in the lungs of control as well as the mice exposed to $SO_2$ with slightly more damage in those exposed to the pollutant. Nasal cavities and trachea appeared normal. Histopathological examination of lung tissues disclosed that several mice in each group had moderately sized focal areas of parabronchial lymphocytic aggregations, comparable to those seen in chronic respiratory disease. These aggregations were considered as background lesions and were not attributed to the $SO_2$ exposure.

## SUMMARY

Single or multiple 3 hr exposures of mice to 13100 $\mu g/m^3$ (5.0 ppm) $SO_2$ alone or in combination with either, carbon particles, zinc sulfate particles of $NO_2$ and $O_3$ mixture had no effect on their susceptibility to streptococcal pneumonia. Continuous exposure for up to 3 month to the same $SO_2$ concentration also had little effect on the susceptibility to respiratory infections as well as other health effect parameters.

## EXPOSURE TO SULFATES

The major thrust of these studies was to determine the effect of a single 3-hr exposure to zinc sulfate ($ZnSO_4$). ammonium sulfate ($(NH_4)_2SO_4$) and zinc ammonium sulfate ($Zn(NH_4)_2(SO_4)_2$ on the susceptibility to streptococcal pneumonia. Exploratory experiments were also conducted to study the effects of air pollutant mixtures containing $ZnSO_4$, $NO_2$ and $O_3$ on susceptibility to respiratory infection.

### Exposure to Individual Sulfates

Mean mortality rates and survival time of mice exposed for 3 hr to various concentrations of the sulfates and subsequently challenged by the respiratory route with *S. pyogenes* are summarized in Table 14. The concentrations of the various sulfates were expressed as $mg/m^3$ of $SO_4$. Although not shown in the table, throughout the replicate experiments, there were no deaths among mice exposed to the sulfates only.

TABLE 14. MORTALITY AND SURVIVAL RATE OF MICE EXPOSED FOR 3 HR TO SULFATES AND CHALLENGED WITH *STREPTOCOCCUS* AEROSOL

| $SO_4$ mg/m$^3$ | $ZnSO_4$ Mortality D/T | $ZnSO_4$ Mortality % | $ZnSO_4$ MST day | $Zn(NH_4)_2(SO_4)_2$ Mortality D/T | $Zn(NH_4)_2(SO_4)_2$ Mortality % | $Zn(NH_4)_2(SO_4)_2$ MST day | $(NH_4)_2SO_4$ Mortality D/T | $(NH_4)_2SO_4$ Mortality % | $(NH_4)_2SO_4$ MST day |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 373/1689 | 22.1 | 12.1 | 165/756 | 21.8 | 12.3 | 233/588 | 39.6 | 10.1 |
| ≤1.1 | 125/599 | 20.9 | 12.2 | 29/120 | 24.2 | 11.9 | 22/48 | 45.8 | 10.1 |
| 1.2-2.0 | 369/813 | 45.4* | 9.1* | 112/445 | 25.2 | 12.0 | 76/191 | 39.8 | 10.7 |
| 2.1-3.0 | 186/278 | 66.9* | 6.2* | 67/192 | 34.9* | 10.9* | 52/96 | 54.2* | 9.2 |
| 3.1-4.0 | - | - | - | - | - | - | 47/144 | 32.6 | 11.2 |
| ≥4.1 | - | - | - | 45/48 | 93.8* | 4.0* | 46/110 | 41.8 | 10.3 |

* Significant change from infected mice exposed to filtered air ($p \leq 0.05$).

Mortality and survival rates were not affected by a 3-hr exposure to 1.1 mg/m$^3$ or lower concentrations of $ZnSO_4$. However, inhalation of 1.3 mg/m$^3$ or higher concentrations resulted in significant excess mortality and reduced survival time. The death rates of control mice challenged with *S. pyogenes* and exposed to filtered air was 22.1% (2.6% S.E.) and among those exposed to 0.5 to 1.1 mg/m$^3$ $ZnSO_4$ 20.9%. Exposure to 1.3 to 1.9 mg/m$^3$ resulted in 23.3% excess mortality and a decrease in survival time of 3 days. Exposure to 2.3 to 2.9 mg/m$^3$ induced 44.8% excess mortality and an approximately 6 day reduction in the mean survival time. The Chi-square ($X^2$) analysis of the results of individual exposure experiments confirmed this dose effect. Within the concentration range of 0.5 to 1.1 mg/m$^2$ $ZnSO_4$ 6 individual test $X^2$ values did not show any significance, whereas 9 out of 10 test $X^2$ values were significant for death rates resulting from exposures to 1.3 to 2.9 mg/m$^3$.

The persistence of $ZnSO_4$ effect on the susceptibility to respiratory infection was studied in a single experiment. Groups of mice were exposed for 3 hr to 1.65 mg/m$^3$ $ZnSO_4$ and 1, 24, and 48 hr later challenged with *S. pyogenes* aerosol. Significant excess mortality (25%) and reduced survival time (2.4 days) was seen at the 1 hr challenge. However, when the infectious challenge was delayed to 24 or 48 hr no differences in mortality or survival time were observed when compared to infected mice exposed to filtered air, thus indicating a recovery from the effect of $ZnSO_4$ inhalation. Scanning electron microscopic observation of tissues of mice exposed to $ZnSO_4$ aerosols and sacrificed 1 and 24-hr later indicated some thickening of the alveolar walls in the lungs and presence of particles in the nasal cavity.

Inhalation of $Zn(NH_4)_2(SO_4)_2$ was less effective in altering the resistance to streptococcal pneumonia. The 3-hr exposure to up to 2.0 mg/m$^3$ had no effect on the resistance to infection. On the other hand, exposures to concentrations above 2.1 mg/m$^3$ significantly enhanced mortality and reduced the survival time. The mean mortality for the control group of mice was 21.8% (2.4% S.E.) and for those exposed to 1.0 to 2.0 mg/m$^3$ $Zn(NH_4)_2(SO_4)_2$ was 25.0%; the corresponding survival times were 12.3 and 12.0 days. The inhalation of 2.1 to 3.0 mg/m$^3$ $Zn(NH_4)_2$ followed by the infectious challenge resulted in 13.1% excess mortality and approximately 1.5 day decrease in survival time. Exposure to 4.1 mg/m$^3$ resulted in 72.0% excess mortality and 8.3 day decrease in mean survival time. The $X^2$ analysis of ten individual exposure expriments confirmed this dose relation, whereas none of the $X^2$ values were significant at concentrations below 2.0 mg/m$^3$, but all four at or above 2.1 mg/m$^3$ were significant.

In general, the exposure to 1.1 to 5.3 mg/m$^3$ $(NH_4)_2SO_4$ was not effective in altering the resistance to streptococcal pneumonia. Although in the concentration range of 2.1 to 3.0 mg/m$^3$ the excess mortality was significant, there was otherwise no apparent relationship between death or survival time and concentrations of the sulfate. The overall mortality among the infected control mice was 39.6 (3.5% S.E.) whereas that of mice exposed to $(NH_4)_2SO_4$ was 41.3%, the corresponding mean survival rates were 10.1 and 10.4 days.

To further assess the effect of exposure to sulfates on the resistance to streptococcal pneumonia the excess mortality and survival time data were subjected to regression analysis. The resulting least square lines are shown in Figures 5 and 6. The effectiveness and dose relation of $ZnSO_4$ in enhancing the mortality caused by the infection is clearly seen. The correlation coefficient was significant for mortality as well as survival time ($r_{(14)}$ = 0.899 and $r_{(14)}$ = 0.891). The estimated concentration of $ZnSO_4$ required to induce 20% excess mortality ($ED_{20}$) was 1.45 mg/m$^3$. The dose response relation for $Zn(NH_4)_2(SO_4)_2$ can also be seen, where the correlation coefficients for mortality and for the survival time were significant ($r_{(12)}$ = 0.983 and $r_{(12)}$ = 0.744). The dose which produced a 20% excess mortality was estimated at 2.4 mg/m$^3$.

Exposure to $ZnSO_4$, $NO_2$ and $O_3$ Mixtures

Changes in mortality among mice exposed for 3 hr to various concentrations of $NO_2$, $O_3$ and $ZnSO_4$ and challenged with *S. pyogenes* aerosol are shown in Table 15. The table also includes estimated excess mortalities, which could be expected to result from a single 3-hr exposure to the individual pollutants in concentration present in the mixtures. These estimates were based on excess mortality data obtained during other studies conducted during the project. With some exceptions the observed excess mortalities were in close agreement with the expected values, suggesting that the inhalation of mixture on air pollutants has an additive effect. However, at the higher concentrations of the pollutants a potentiation appeared to be present resulting in excess mortalities approximately twice as high as those expected.

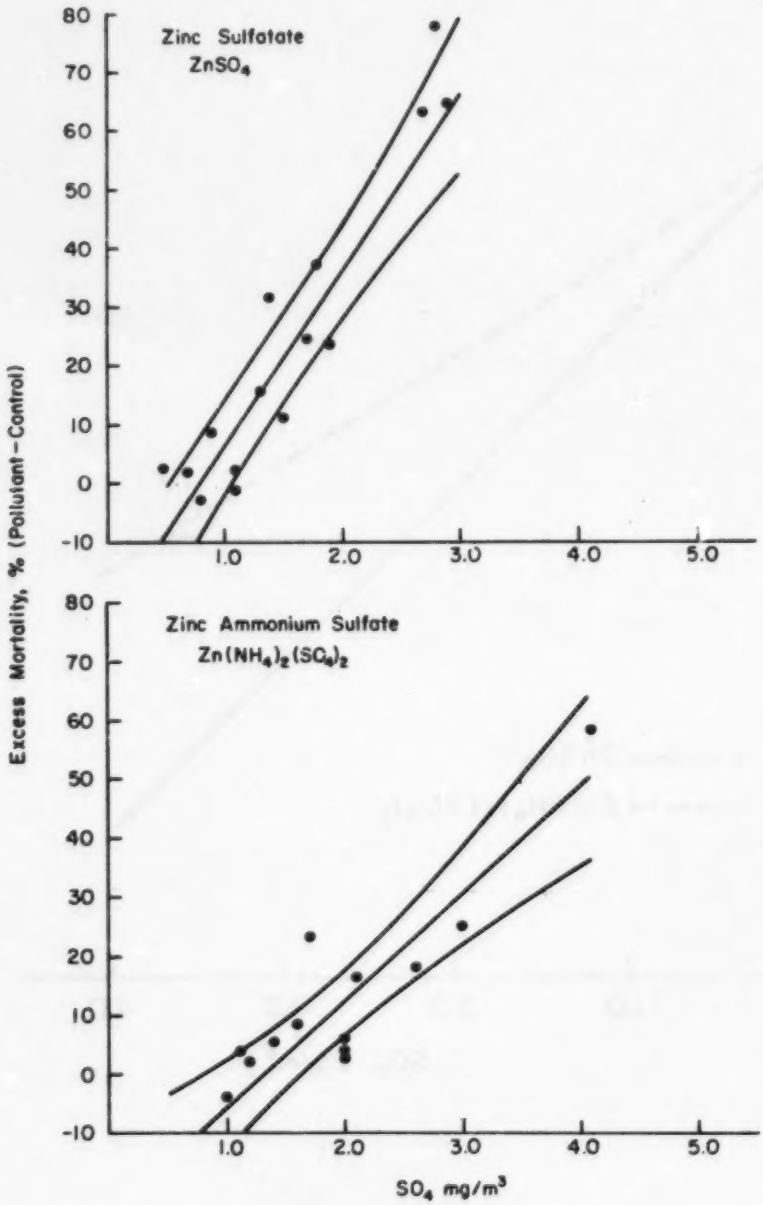


**Figure 5. Excess mortality in mice exposed for 3 hr to sulfates and challenged with Streptococcus aerosol.**

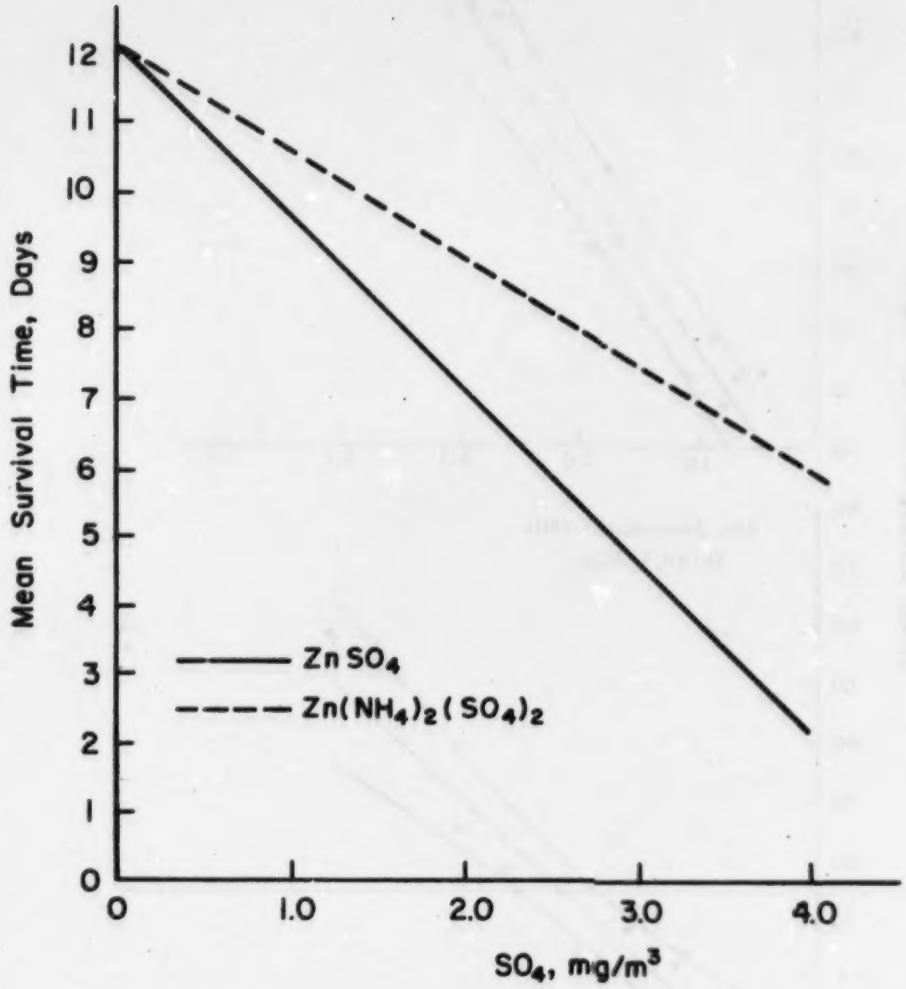


Figure 6. Effect of 3 hr exposure to sulfates on the surival of mice infected with Streptococcus aerosol.

TABLE 15. MORTALITY OF MICE EXPOSED FOR 3 HR TO $ZnSO_4$ AND $NO_2$ AND $O_3$ MIXTURES AND CHALLENGED WITH *STREPTOCOCCUS* AEROSOL

| Concn., $\mu g/m^3$ | | | Mortality | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Air | | Pollutant | | Change,% | |
| $ZnSO_4$ | $NO_2$ | $O_3$ | D/T | % | D/T | % | Actual | Expected[a] |
| 400 | 940 | 98 | 26/72 | 36.1 | 31/72 | 43.1 | 7.0 | 8.0 |
| 800 | 2820 | 98 | 34/120 | 28.3 | 41/120 | 34.2 | 5.9 | 0.6 |
| 1100 | 3760 | 196 | 12/144 | 8.3 | 38/144 | 26.4 | 18.1* | 20.5 |
| 1200 | 2820 | 98 | 8/144 | 5.6 | 55/144 | 38.2 | 32.6* | 18.7 |
| 1400 | 940 | 98 | 13/72 | 18.1 | 40/72 | 55.6 | 37.5* | 36.6 |
| 1400 | 2820 | 98 | 8/96 | 8.3 | 79/96 | 82.3 | 74.0* | 34.9 |
| 1500 | 3760 | 198 | 8/96 | 8.3 | 81/96 | 84.4 | 76.1* | 32.1 |

* Significant change from corresponding infected mice exposed to filtered air ($p \leq 0.05$).

[a] Expected change in mortality based on exposure to the individual pollutants in concentrations present in the mixture.

SUMMARY

Inhalation of $ZnSO_4$ (>1.2 mg/$m^3$) or $Zn(NH_4)_2(SO_4)_2$ (>2.1 mg/$m^3$) followed by a respiratory challenge with airborne *S. pyogenes* resulted in significant excess mortality and reduced survival time in mice. For the 3 hr inhalation exposure the estimated concentration of $ZnSO_4$ which induced 20% excess mortality ($ED_{20}$) was 1.45 mg/$m^3$ whereas that of $Zn(NH_4)_2$ was 2.40 mg/$m^3$. Exposure to $(NH_4)_2SO_4$ aerosol in concentrations ranging from 1.1 to 5.3 mg/$m^3$ had no effect on the susceptibility to the respiratory infection.

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